

**FLAVOUR REFORMULATION  
AND FLAVOUR STABILITY**

by

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## **ABSTRACT**

This project addressed two areas in the flavour industry – flavour reformulation required as a consequence of ingredient changes and flavour stability in solid food products.

The aim of the flavour reformulation study was to evaluate both instrumental and modelling approaches to reformulate commercial flavourings for food products of different fat contents. A strawberry flavouring was chosen, which delivered an acceptable flavour in pectin jelly (0% fat) but did not perform so well in chewy candy (8% fat). Aroma release profiles in people eating these two sweets were measured by APCI-MS, the relative release differences for each flavour compound was determined and then used to reformulate the strawberry flavouring so its release in both sweets was similar. The sensory performance of candies confirmed a significant difference between the reformulated and original flavour ( $p < 0.05$ ). The modelling approach was based on compound hydrophobicity (Log P) and the fat content of the food. This was used to estimate relative differences in flavour delivery for products with two varying fat contents. Flavourings were reformulated for yoghurt with reduce fat level, and the measured results showed good correlation with model's prediction ( $r = 0.95$ ).

The aim of the flavour stability study was to explore the impact of product storage and flavour solvent in biscuits with vanilla flavouring. After eight weeks storage at 45 °C, up to 20% vanillin loss was measured, but sensory results suggested no difference in vanilla flavour perception. Texture analysis indicated that biscuits using triacetin (TA) as flavour solvent were more brittle than biscuits made with propylene glycol (PG). This was explained by X-ray CT analysis results which showed TA biscuits had larger pores than PG biscuits. Additionally, TA solvent



provided higher vanillin stability during storage, so it should be a better choice than PG solvent used in flavourings for biscuits.

### **IMPACT STATEMENT**

This research proved that it is feasible to apply laboratory-derived knowledge and adapt scientific techniques in a commercial context. It also demonstrated how the research findings can be transferred into the commercial field through two studies.

The first study successfully illustrated two novel approaches to reformulate flavourings between food products of differing fat contents. Comparing with traditional trial and error process, this study demonstrated that instrumental and modelling approaches can be more effective ways for flavour reformulation in the flavour and food industry. The cost of flavour reformulation including the reformulation time and the cost in-use can therefore be reduced considerably. The results of this study have been presented to company's major clients worldwide, and this has been applied commercially in a range of products including the successful reformulation of a soft drink (£50 M brand value 2010) into new formats (2011).

The second study increased the understanding of the flavour stability within the stored food products. This ensured the flavour company to deliver high quality materials not only to their clients before food manufacturing, but also maintained its quality in the food products during storage for their end users. Comparing two flavour solvents – propylene glycol and triacetin, the results of this study suggested that triacetin is a better solvent as it offered better flavour stability in the stored biscuits. The findings can also be applied to reduce the cost in-use for vanilla flavouring in biscuit, which is especially beneficial for food and flavour company when high cost natural vanilla flavourings are required.

## PUBLICATIONS

As the result of flavour reformulation studies, three papers have been published:

1. LINFORTH, R., CABANNES, M., HEWSON, L., YANG, N. and TAYLOR, A. (2010). Effect of Fat Content on Flavor Delivery during Consumption: an in Vivo Model. *Journal of Agricultural and Food Chemistry*, 58 (11), 6905-6911
2. YANG, N., LINFORTH, R. S. T., WALSH, S., BROWN, K., HORT, J. and TAYLOR, A. J. (2011). Feasibility of reformulating flavours between food products using in vivo aroma comparisons. *Flavour and Fragrance Journal*, 26 (2), 107-11
3. LINFORTH, R., BLEE, N., YANG, N., BROWN, K. and TAYLOR, A. (2011). Variation in aroma release between panellists consuming different types of confectionary. *Flavour and Fragrance Journal*, 26 (3), 186-191

Two conference abstracts have been accepted:

1. Reformulating flavours using instrumental analysis (2010). Abstract and oral presentation in the 239th American Chemistry Society National Meeting, San Francisco, USA.
2. Impact of flavour carrier solvent on vanillin stability and the aroma of shortcake biscuits (2011). Abstract for poster presentation in the 13th Weurman Flavour Research Symposium, Zaragoza, Spain.

Another two papers are being prepared for publication:

1. Impact of flavour solvent on vanillin stability, biscuit texture and flavour generation in shortcake biscuits during storage. Aimed at *Journal of Agricultural and Food Chemistry*.
2. Impact of flavour solvent on matrix micro-structure and chemical stability of aroma compounds. Aimed at *Flavour*.

## **PREFACE**

This PhD project was funded by the Knowledge Transfer Partnership (KTP) programme, which is a UK government funded programme to help small to medium sized companies increase their competitiveness by collaborating with academic institutes. Aromco Ltd, a flavour house in the UK, was the company partner in this programme and the flavour group, a sub team of the division of food sciences at the University of Nottingham were the academic partners. Two research themes were developed to investigate major challenges in the flavour industry that could benefit from academic involvement: flavour reformulation and flavour stability. The study on flavour reformulation was supervised by Prof. Taylor and Dr. Linforth, and the flavour stability study was supervised by Dr. Hort and Dr. Fisk.

This thesis is presented in four parts (I-IV). Part I ‘Introduction’ contains Chapter 1 as an overall introduction reviewing some fundamental theories on flavour release and flavour stability. Part II ‘Flavour Reformulation’ consists of three chapters (2-4). Chapter 2 covers introduction to the potential approaches of flavour reformulation plus research objectives. Chapter 3 describes the materials and methods employed in this study, and Chapter 4 presents the results obtained and corresponding discussions. Part III ‘Flavour Stability’ follows the same pattern as Part II, including Chapter 5 (Introduction), Chapter 6 (Materials and Methods), and Chapter 7 (Results and Discussions). Finally, Part IV contains Chapter 8 which provides an overall summary of the research outcomes from both research themes and their commercial applications.

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## LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
APCI-MS	Atmospheric Pressure Chemical Ionisation-Mass Spectrometry
ASL	Accelerated Shelf Life
AV	Average
CT	Computed Tomography
CV	Coefficient of Variation
EE	Extraction Efficiency
FC	Fat Content
GC-MS	Gas Chromatography-Mass Spectrometry
HF	High Fat
HMF	5-hydroxymethyl-furfural
HPLC	High Performance Liquid Chromatography
HS	Headspace
Imax	Maximum Ion Intensity
IS	Internal Standard
K <sub>aw</sub>	Air-water Partitioning Coefficient
KTP4	Confidential Formula Coding
LE	Lipid Effect
LF	Low Fat
MANOVA	Multivariate Analysis of Variance
MCT	Multiple Comparison Test
MW	Molecular Weight
PA	Peak Area
PG	Propylene Glycol
ppb	Parts Per Billion
ppm	Parts Per Million
QSPR	Quantitative Structure-Property Relationship
SE	Standard Error
STD	Standard Deviation
TA	Triacetin
VP	Vapour Pressure
w/w	Weight by weight

# **PART I INTRODUCTION**

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## **1 INTRODUCTION**

### **1.1 PROJECT BACKGROUND**

The increasing demands from consumers for healthy low fat products require food companies to produce foods with reduced amounts of fat. Although removing fat creates flavour imbalance (Taylor & Hort, 2007), consumers expect the flavour perceived in the low fat version to be the same as its original version. The food companies pass this pressure to their flavour suppliers to provide reformulated flavourings that work in low fat products. Any flavour company who can successfully reformulate these required flavourings for food companies will gain more share in the global flavour market which has an estimated sales volume of \$22,000 million in 2010 (Leffingwell&Associates, 2011).

In the flavour industry, the traditional procedure of flavour reformulation is done by trial and error through a process of panel testing and continuous modification and repeated evaluation by the flavourists and food application technologists. This can be a very time-consuming and costly method, so it is important for flavour companies to gain a better understanding of how flavour performs in foods and is perceived by consumers, allowing new products to be developed more quickly and effectively. The knowledge about flavour release concepts and analysis techniques was reviewed in the following (Section 1.2).

Comparing with traditional method, it is also essential to develop some alternative ways to reformulate flavourings. Two novel approaches were proposed as using

instrumental analysis and mathematical modelling to guide flavour reformulation. These were the first research theme involved in the Part II of the thesis.

On the other hand, consumers expect good flavour quality to be maintained during shelf-life, although flavourings are liable to change due to many factors (reviewed in Section 1.3). Therefore, flavour stability is another critical issue in the food and flavour industry. In general, liquid flavourings, as one of the most common types, have a shelf-life of at least six months to one year, but their stability within the food product is not well established. They are normally made by mixing aroma compounds with a particular flavour solvent. The use of different flavour solvents is determined by its cost and application, but their effects on flavour stability and performance within the food products are not well understood. Thus, the second research theme was focused on flavour stability within food products and the comparison of two flavour solvent systems, discussed in Part III of the thesis.

Generally, the nature of the project determined that plenty of practices were applied into real food products in order to reveal the feasibility of transferring scientific findings into real business. The types of food products covered in this thesis were confectionery, yoghurt and bakery products. These products were developed by Aromco to imitate the market products at a small-scaled bench production. Dealing with the complex matrices in real food in this study could cause more variations than a simple experimental composition. Therefore, it was necessary to evaluate potential variations from the above three food products during preliminary studies in order to maintain the quality of research involved in both research schemes.

## **1.2 FLAVOUR RELEASE**

### **1.2.1 Flavour Definition**

The overall flavour of a food is generally agreed to consist of taste, aroma and trigeminal sensations (Fisher & Scott, 1997): taste is imparted by non-volatile components during eating and is perceived by the tongue; aroma is imparted by volatile components and is perceived by the olfactory receptors in the nose; trigeminal refers to tactile and temperature responses during chewing.

Among the three sensations, aroma perception of a food is considered as a very important aspect that influences consumers' acceptability and the enjoyment of eating (Bakker et al., 1996). In the context of the work by Taylor (1998), aroma plays the major role in determining overall flavour. Therefore, in this thesis, the use of the term flavour relates to the aroma compounds unless specified. For aroma sensation to occur, an aromatic compound must be sufficiently volatile to allow detection at distance (Fisher & Scott, 1997). Therefore, aroma compounds are also referred to as volatiles in some context.

### **1.2.2 Flavour Release & Perception**

Even before the food is placed in the mouth, aroma compounds can enter the nasal cavity to make contact with the receptor cells in the olfactory epithelium through the orthonasal route (Figure 1.1). This is especially the case before drinking coffee or wine, where a great deal of information can be obtained about the product quality from just sniffing it. During drinking or eating, the volatile compounds released from food enter the air in the mouth and are then transported to the nose via the retro-nasal route (Figure 1.1). Some of the volatiles will reach the olfactory receptors either by the chewing action which pumps some air into the

throat, or by swallowing when a large volume of air is transferred. Aroma compounds reaching the olfactory centre interact with odour proteins to trigger olfactory transduction (Laing & Jinks, 1996).

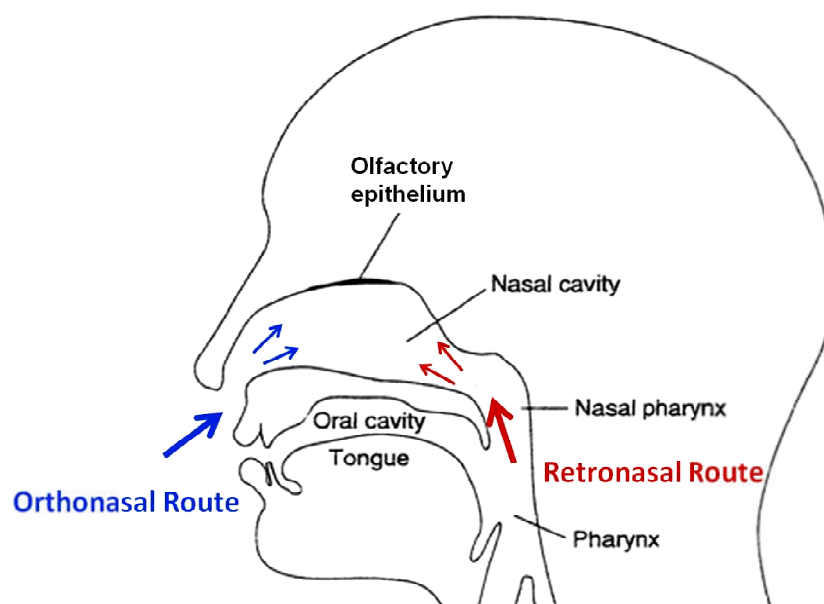


Figure 1.1 Diagrammatic representation of olfactory perception through orthonasal route and retronasal route, adapted from (Goldstein, 1999)

Additional to the understanding of the olfactory physiology for flavour perception, the theory of distal and proximal stimuli is also important. Its application to food flavour perception is illustrated in Figure 1.2.

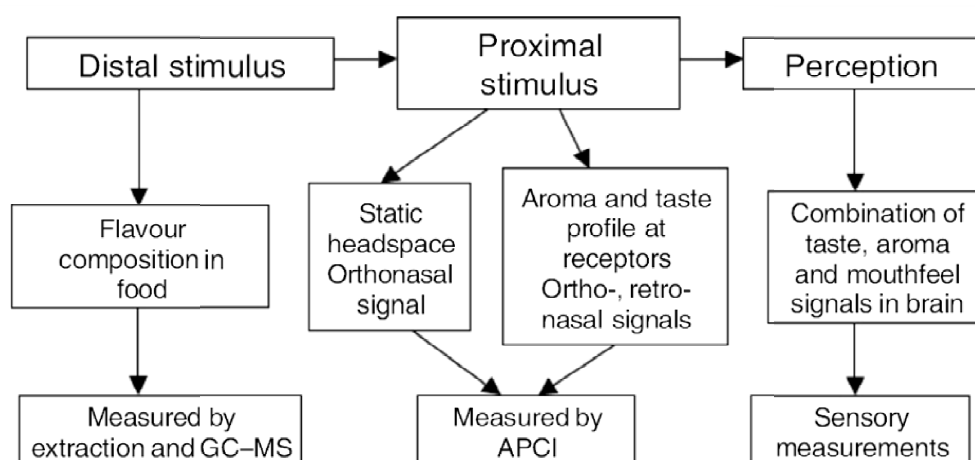


Figure 1.2 Relationship between distal and proximal stimulus (top row), flavour-sampling method (middle row) and flavour analyses (bottom row), demonstrated by Taylor and Hort (2004)

As illustrated in Figure 1.2, the distal stimulus is the flavour composition in the food, measured by gas chromatography-mass spectrometry (GC-MS); while the proximal stimulus represents the flavour profile that could be sensed by both orthonasal and retronasal route. The orthonasal proximal stimulus could be indicated by the volatile content in the gas phase above the food; whereas, retronasal proximal stimulus requires the flavour profile to be measured close to the receptors. Both orthonasal and retronasal proximal stimulus could be measured by APCI-MS technique, which is described in Section 1.2.4. Perception can be measured by various sensory techniques.

In this thesis, the reformulation study focused on the proximal stimulus and perception, so APCI-MS and sensory measurements were applied. The flavour stability study applied GC-MS and HPLC techniques to measure the distal stimulus along with relevant sensory analysis.

### 1.2.3 Factors Affecting Flavour Release

A number of factors influence flavour release and perception, including

- i) thermodynamic factors (e.g., partitioning coefficient) and kinetic factors (e.g., diffusion and mass transfer) that control flavour release;
- ii) the physicochemical properties of aroma compounds that are related to their availability for perception (e.g., volatility and hydrophobicity);
- iii) effect of major food components (e.g., lipid, protein, carbohydrate) that interact with the aroma compounds ;
- iv) oral processing of food that take physiological factors into account (e.g., chewing rate, saliva flow, swallow frequency).

The following section discusses each factor in more detail.

#### *1.2.3.1 Effect of thermodynamic & kinetic factors*

Aroma release from foods during eating is a dynamic process which is determined by thermodynamic and kinetic factors illustrated by two main factors: partition phenomena and mass transport (de Roos & Wolswinkel, 1994).

Partition is a fundamental parameter describing the distribution of a volatile compound between two phases at equilibrium, so it has been widely studied. Aroma released from foods under equilibrium conditions depends on the distribution (partitioning) of aroma molecules between air and food phases (Taylor, 1999). If a thermodynamic equilibrium exists between a product and a gas phase, the concentration of flavour component ( $C_a$ ) in the air is related to the air-product partition coefficient ( $K_{ap}$ ), expressed in the Equation 1.1.

$$K_{ap} = \frac{C_a}{C_p} \quad \text{(Equation 1.1)}$$

The application of this partition coefficient to food systems can be complicated by the fact that foods generally contain several phases. Similar principles can be applied to other phases, such as oil-water ( $K_{ow}$ ) and air-water ( $K_{aw}$ ) partition, which are important parameters involved in this study.

Partition values mentioned above represent the distribution of a volatile compound between two well defined phases in a system at equilibrium. However, eating is a dynamic process under the non-equilibrium conditions, which considered the driving force for transfer aroma compounds across the interface as the flavour concentration difference between product and gas phase (Taylor, 1996).



Several mathematical models derived for predicting flavour release under dynamic conditions were reviewed by van Ruth and Roozen (2010). These models illustrated that different mechanisms of mass transport were applied to different types of food products. Although many factors influencing flavour release can be determined, flavour release is still complex and difficult to predict, owing to the rapid changing conditions during eating. Le Thanh et al. (1992) described two types of interactions between flavour compounds and major food components: the attractive interactions involve fixation of flavour compounds on food components, whereas the repulsive interactions concern the release of aroma compounds. The nature of these interactions depends on the physicochemical properties of the compounds and the food matrix, which will be reviewed in the following two sections.

#### *1.2.3.2 Effect of physiochemical properties of aroma compounds*

Since flavour release depends on the availability of the flavour compounds in the gas phase and the affinity of the flavour compounds for the food matrix, Kinsella (1989) stated that the interactions with food components could be determined by various physicochemical properties of flavour compounds, such as molecular weight, vapour pressure, boiling point and hydrophobicity. In this study, volatility and hydrophobicity are the two key factors involved.

The volatility of flavour molecules at a given temperature is important for transport and as a result for the compound concentration at the olfactory epithelium. The vapour pressure (VP) of the flavour compounds is an indicator of volatility at a given temperature, which is important for transport and as a result for the compound concentration at the olfactory epithelium. However it is difficult

to determinate VP at a lower temperature such as ambient or body temperature (Boelens, 1986). Several scientists have tried to determine the volatility of the compounds based on experimental data in model systems (Roberts & Acree, 1996) or predict these physicochemical parameters by modelling (Carey et al., 2002).

Taylor (1998) reviewed some principle physicochemical parameters for the volatile flavour compounds and highlighted the essential parameter for the hydrophobicity of molecules - Log P. It is the logarithm of the partition coefficient of the compound between octanol-1 and water, as shown in the Equation 1.2.

$$\text{Log } P = \text{Log } K_{ow} = \text{Log } \frac{C_{octanol}}{C_{water}} \quad (\text{Equation 1.2})$$

At equilibrium, the concentration of flavour component in the octanol ( $C_{octanol}$ ) divided by its concentration in water ( $C_{water}$ ) gives the octanol/water partition coefficient ( $K_{ow}$ ), which is considered to be a reasonable approximation to the oil/water partition coefficient (Shiu & Mackay, 1986). Log P value is a well-accepted indicator of the polarity of compounds.

Shojaei et al. (2006a) estimated Log P using four types of software for 20 volatile compounds, and the EPI (Estimation Programs Interface) Suite<sup>TM</sup>, as the physical/chemical property estimation programme, was found to correlate best with the experimental Log  $K_{ow}$  values. So EPIsuite<sup>TM</sup> that estimates the Log P value using an atom/fragment contribution method was used in this study. EPIsuite<sup>TM</sup> can also provide estimated vapour pressure (VP) values using a combination of techniques. Figure 1.3 shows the estimated VP (mm Hg, at 25 °C) and Log P value with 40 aroma compounds involved in this study as an example.

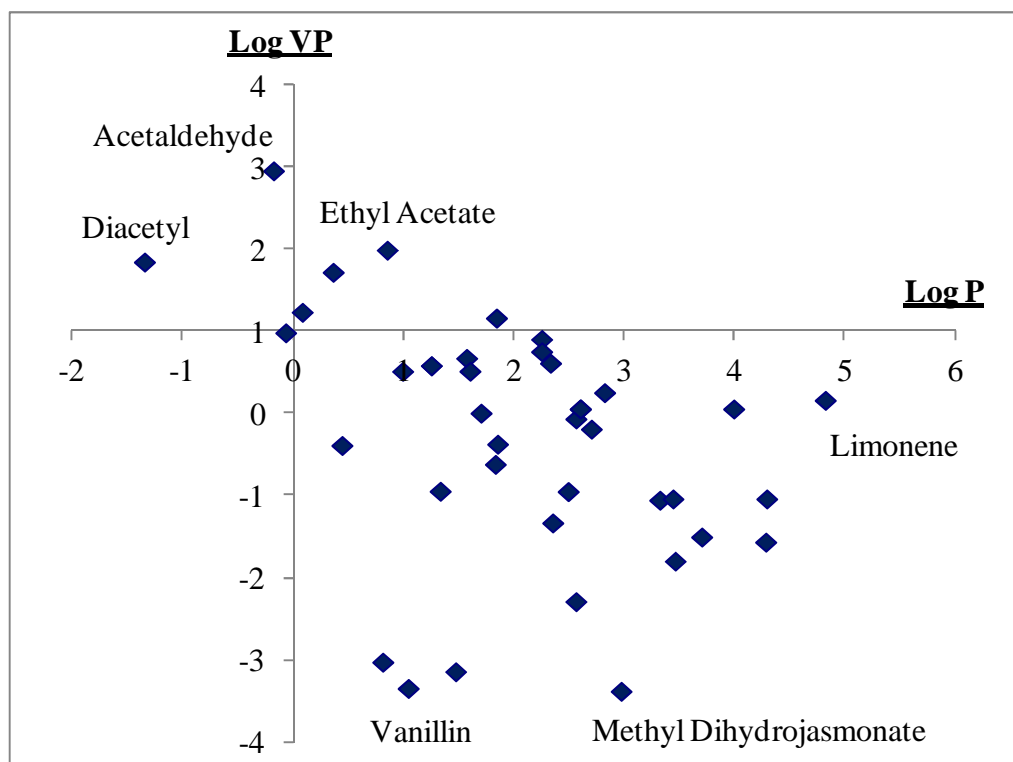


Figure 1.3 Plot of Log P (hydrophobicity) against Log VP (volatility) of 40 aroma compounds

The distribution of aroma compounds (Figure 1.3) shows the extremes boundaries by compounds like acetaldehyde and diacetyl (high volatility and hydrophilic); vanillin and methyl dihydrojasmonate (low volatility and medium hydrophobic); limonene (medium volatility but very hydrophobic). In addition, the relationship between the hydrophobicity (Log P) and volatility (Log VP) was indicated that the volatility of compounds decreases with the increase of hydrophobicity, that is, more hydrophobic compounds may be less volatile. However, this is not always true, such as two compounds with similar hydrophobicity but one (e.g. vanillin) is much less volatile than another (e.g. ethyl acetate). Moreover, compounds with similar volatility (like vanillin and hedione) may also differ in their hydrophobic properties.

Additionally, physicochemical interactions can change flavour intensity or even generate new flavours. The use of the term ‘matrix’ expresses the bulk of the

sample that contains a mixture of volatile compounds and the ‘matrix effect’ refers to combined effects of all components of the sample. The interaction of the matrix components (e.g. flavour solvent) with aroma compounds influence its solubility and partition coefficient, which are also important factors in controlling flavour release during food consumption. More details on flavour solvent and aroma compound interactions are reviewed in Section 1.3.2 .

### *1.2.3.3 Effect of major food components*

Food flavours can be dissolved, bound, adsorbed, entrapped, encapsulated or diffusion limited by food ingredients (Kinsella, 1989). Flavour perception in food is highly influenced by interactions between volatile aroma compounds with a range of non-flavour food matrix components (Bakker et al., 1996). The interactions between flavour compounds and three major food ingredients (lipids, proteins, carbohydrates) are summarised in Table 1.1 and discussed respectively further.

*Table 1.1. Types of interactions that may occur between flavour and food components (Kinsella, 1989)*

<b>Component</b>	<b>Possible Interactions</b>
• Lipids	<ul style="list-style-type: none"> <li>- solution</li> <li>- dispersion</li> <li>- adsorption</li> <li>- entrapment</li> </ul>
• Proteins	<ul style="list-style-type: none"> <li>- adsorption</li> <li>- absorption</li> <li>- specific binding</li> <li>- covalent interactions</li> <li>- entrapment</li> <li>- encapsulation</li> </ul>
• Carbohydrates	<ul style="list-style-type: none"> <li>- adsorption</li> <li>- entrapment</li> <li>- complexation</li> <li>- encapsulation</li> <li>- viscosity/diffusion limitations</li> </ul>

Adsorption and absorption are types of binding specific for low-moisture food systems (such as a biscuit) which consists of an outer surface and inner surface with fine pores and channels. Volatile compounds can therefore be sorbed onto both the outer and the inner surface (adsorption). The aroma compound may also 'dissolve' in the material of the particle (absorption). The composition of the food matrix determines the extent and type of aroma binding.

#### *A) Lipid-flavour interactions*

It is generally acknowledged that flavour-lipid interactions are one of the most important factors to affect the release and perception of aroma compounds (Ollivon, 2006), and these interactions were a major concern during the flavour reformulation study in this thesis. In products containing fat, hydrophobic aroma compounds are bound to fat molecules by weak, reversible, Van der Waals and hydrophobic interactions. The nature of the aroma compounds has a big impact on its behaviour towards the matrix. Brauss et al. (1999b) reported that more hydrophobic compounds with higher Log P, e.g. anethole (Log P = 3.45) and terpinolene (Log P = 4.44), were influenced most profoundly by changes of fat content in yoghurt.

In fact, lipids adsorb and solubilise hydrophobic flavour compounds and reduce their vapour pressures. Small amounts of oil can significantly decrease the headspace flavour concentration. Weel et al. (2004) showed the minimum effective oil content for ethyl hexanoate was less than 0.01% from static headspace measurement, 0.5-1% from orthonasal aroma perception (two trained panellists) and 1% from the retronasal perception. Many studies have (de Roos & Wolswinkel, 1994; Doyen et al., 2001; Roberts et al., 2003) shown that the effect

of oil content on in-vivo aroma release or perception is smaller than expected from equilibrium headspace studies. Linforth et al. (2002) explained the differences observed between static headspace and in-vivo delivery are due to the dynamic nature of in-vivo delivery affecting mass transfer.

The effects of lipids on the rate and amount of the aroma released have been reviewed extensively (de Roos, 1997). Plug and Haring (1993) stated that changing the fat content of foods would change volatile intensity and the rate of release. However, Brauss et al. (1999b) illustrated that there were no changes in rates of release in-vivo but the maximum volatile intensity of aroma compounds was affected by the fat content in yoghurt.

Changing the fat content of food may not only change the partition of aromas that lead to imbalanced flavour, but also change the viscosity and mouthfeel of food. The importance of fat in the perception of food has been reviewed previously (Hort & Cook, 2007). Additionally, fat may also trigger a specific fat receptor in the palate (Mattes, 2003), it can also act as a precursor for certain flavours which would not be formed in its absence, such as those observed during Maillard reaction (Elmore et al., 2002). Therefore, fat has a significant effect on food acceptability and formulating flavourings for food with different fat content could be challenging.

### ***(B) Protein-flavour interactions***

Generally, there are two major interactions concerning proteins and flavour molecules: 1) reversible binding (i.e., physical adsorption through Van der Waals forces or hydrophobic interactions between hydrophobic regions of proteins and volatiles); 2) irreversible binding (i.e., chemical reactions of various strengths).

The strength of the interactions depends on the ability of flavour volatiles to induce the unfolding of the protein. The interactions also depend on the specific nature of the volatile compounds, for example, aldehydes are particularly reactive towards proteins by forming stronger bound than alcohols or ketones (Damodaran & Kinsella, 1981).

Reineccius (2006b) concluded that these interactions could be influenced by the type and amount of protein, type of flavouring components, the physicochemical conditions and the composition of a food system (e.g., pH, temperature, time, ionic strength, water content). Most intensively studied proteins with respect to flavour-protein interactions are  $\beta$ -lactoglobulin (Charles et al., 1996; Andriot et al., 1999), albumin (Damodaran & Kinsella, 1980; Burova et al., 1999) and soy protein (ONeill & Kinsella, 1987; OKeefe et al., 1991).

Obviously, when the aroma compound has been bound or lost permanently, this consequent binding may affect the flavour balance of the food. The addition of proteins to a water-aroma compound model system lowered the concentration of the volatile components in the headspace (Schirle-Keller et al., 1992). Hansen and Heinis (1992) have observed a decrease of benzaldehyde and limonene flavour intensity in presence of whey proteins or casein. Proteins also caused a decrease in the volatility of flavour compounds (Charles et al., 1996).

### ***(C) Carbohydrate-flavour interactions***

Carbohydrates are thought to have a lesser role to play in flavour interaction (Delarue & Giampaoli, 2006). They are classified as monosaccharides, disaccharides and polysaccharides, which can affect the flavour perception in different ways. Monosaccharides and disaccharides (such as glucose, fructose,

sucrose, lactose) could be defined as simple sugars, which may increase the vapour pressure for certain volatile compounds and a marked decrease for others, especially hydrophobic aroma compounds (Nahon et al., 1998). It is generally considered that the addition of sweeteners to the food system enhances the perceived flavour, but any changes in a formulation that affect sugar content may also have profound effects on the release of the aroma compounds in that food.

Pectin gels consist of polysaccharide networks surrounded by water, which can prevent the release of aroma molecules from the gel to the gas phase above. Hansson et al. (2002) found aroma concentration in the headspace was related to gel texture, i.e., stronger gels provided low aroma concentration, due to entrapment of aroma molecules within the gel structure. Therefore, the strength of the gel remained consistent in this study.

Kant et al. (2003) found aroma compounds were retained by starch with reversibility, so volatile release was greater during consumption than expected based on static headspace data. Tietz et al. (2008) concluded that the formation of starch-aroma complexes influences aroma release as the helical structures consist of hydroxyl groups oriented to the outside of the coil and a central hydrophobic region that retain the hydrophobic flavour compounds and fats. Therefore, starch being the major ingredient for a biscuit (Chapter 6) might play an important role during flavour stability studies.

#### *1.2.3.4 Effect of food oral process*

Food in the mouth undergoes both chemical and physical changes, which affect flavour release. The physical changes in the mouth include temperature change, hydration of dry foods, mixing of food phases and the effect of bolus size. Some



examples of chemical changes in the mouth are reactions between salivary components (mucin, amylase). E.g., saliva can interact with some aroma compounds causing a change in release and perceived flavour (Roberts & Acree, 1995; van Ruth et al., 1995; van Ruth & Buhr, 2003).

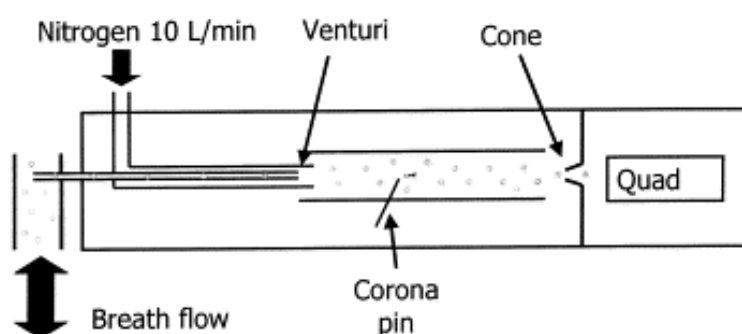
Variations in oral physiological movements are widely reported in the literature. The source of variations could be generally divided into i) individual difference in physiology; ii) food properties differences.

Indeed, humans vary considerably in their anatomy (mouth volume, mucus layer thickness) and their physiology (rate of breathing, saliva flow rate, and so on). The impact of intra and inter-subject oral physiological variance has been well studied, and several studies indicated that the inter-subject variance was greater than intra-subject variation (Brown et al., 1994; Kemsley et al., 2002). The release of aroma compounds have been shown to be dependent on chewing frequency, chewing number and masticatory muscle activity (Haahr et al., 2004; Pionnier et al., 2004). The perception of flavour has also been shown to correspond with the variation in volatile release caused by changes in oral physiological movements (Mestres et al., 2006).

On the other hand, different food properties, especially the effect of texture has been well studied on its influence of oral physiology movements (Aprea et al., 2006; Blissett et al., 2006), and the consequent impact on release of aroma compounds and the perception of flavour perception (Baek et al., 1999; Weel et al., 2002). Using model gel systems, Weel et al. (2002) pointed out the perception of flavour did not appear directly related to in-vivo aroma concentration, but was determined by the gel texture.

#### 1.2.4 Flavour Release Measurement

Measuring the orthonasal aroma release can help us identify the flavour profile from intact food prior to eating, e.g. sniffing a cup of coffee or opening a bag of crisps. The static headspace measurements can be applied for orthonasal aroma release measurement. However, measuring the flavour changes during food oral processing through the retronasal route is more challenging. Previous attempts at flavour release measurements were reviewed with varying degrees of success (Taylor & Linforth, 1994). The technique of Atmospheric Pressure Chemical Ionisation Mass Spectrometry (APCI-MS) had been successfully adapted by Linforth and Taylor (1998) to measure the volatile content of expired air from people during the consumption of food. The related commercially available equipment is called MS-Nose (Micromass, Manchester, UK). A key feature of this technique is a particular interface (Figure 1.4), which uses a venturi to sample air from the nose, mouth or the headspace above a food at a sampling flow rate of 5 to 50 mL/min (Taylor & Linforth, 2000).



*Figure 1.4 Diagrammatic illustration of the APCI-MS interface, demonstrated by Taylor and Linforth (2000)*

APCI-MS is a 'soft' ionisation method based on proton transfer reaction. Water molecules are ionised by accepting protons ( $H^+$ ) when they enter the source of the MS and form the hydronium ion ( $H_3O^+$ ).  $H_3O^+$  does not react with any of the natural components of air (such as nitrogen, oxygen and carbon dioxide) that have

proton affinities lower than H<sub>2</sub>O, but most common aroma compounds have proton affinities larger than H<sub>2</sub>O, so H<sub>3</sub>O<sup>+</sup> will collide with the aroma compounds of interest to transfer the proton and then ionise the aroma molecule. For each compound, the ionisation parameters (positive or negative ionisation, cone voltage) are optimised so that fragmentation is either minimised or controlled, that is why it is known as a 'soft' ionisation process compared to Electron Impact ionisation. Indeed, this technique produces mainly molecular ions by addition of a proton (H<sup>+</sup>) in positive ionisation mode to give RH<sup>+</sup> (H<sub>3</sub>O<sup>+</sup> + R  $\longrightarrow$  H<sub>2</sub>O + RH<sup>+</sup>), where R represents an aroma molecule.

The RH<sup>+</sup> ions enter the Quadrupole Mass Analyser, where they are measured and detected on the basis of their mass to charge (m/z value). The mass spectrometer can be operated in Full Scan Mode to identify significant ions, and if the key volatile components are known, the mass spectrometer is set in the Selected Ion Mode to increase the sensitivity. However, the identification of compounds depends entirely on mass resolution, so neither structural isomers nor stereoisomers can be identified.

The sensitivity of APCI-MS technique is typically around 10 ppbv (nL/L) for most masses, and can be detected down to 100 pptv (pL/L) for others (Taylor & Linfoth, 2000). The odour detection threshold is defined as the minimum concentration of a substance that can be detected by the human nose, and several values were summarised or predicted in the literature (Van Gemert & Nettenbreijer, 1977; Marin et al., 1988; Abraham et al., 2002). The thresholds for aroma compounds vary from a few parts per trillion by volume (pptv, pL of aroma per L air), through parts per billion (ppbv, nL/L) to parts per million (ppmv,  $\mu$ L/L).

Therefore, the APCI-MS technique can detect the majority of aromas close to their odour threshold, and is sufficiently sensitive for use in a range of foods from pectin/ gelatine gel (Linforth et al., 1999), to biscuit (Brauss et al., 2000) and beer (Taylor et al., 2010), just to name a few. However, APCI-MS is not sensitive enough to meet odour threshold values of all compounds, and some compounds fragment badly, such as furaneol, which creates noise and confusion in the system.

Another commonly used mass spectroscopic method is proton-transfer-reaction mass spectrometry (PTR-MS) firstly introduced by Lindinger and co-workers (1993), and recently reviewed by Blake et al. (2009). The key differences between APCI-MS and PTR-MS are that the later technique could form the reagent ions separately and the reaction between reagent ions and volatile compounds is controlled in a drift tube at a precise temperature and pressure. This gives PTR-MS a lower background count and higher sensitivity, but it spends more time to collect signal for a particular ion. Despite the fact that both APCI and PTR have limitations to differentiate the compounds with the same molecular mass, both techniques have contributed much to our understanding of flavour release from food matrices (van Ruth et al., 2003; Biasioli et al., 2011a).

Other methods for real-time flavour release analysis are also available. Smith and Spanel (1996) have developed a method based on SIFT (Selected-Ion Flow Tube) and reviewed recently (Spanel & Smith, 2011). This technique is programmed to deliver not only  $\text{H}_3\text{O}^+$  but also  $\text{NO}^+$  and  $\text{O}_2^+$  based on the different reaction mechanisms of the reagent ions. SIFT provides softer ionisation than PTR, but more complex set-up and more difficulty of interpreting data comparing to APCI. Additionally, the limit of detection is compound-dependent and may not be an

ideal tool for breath-by-breath analysis of the mixture when the complex ion spectra formed from the different ionisation pathways (Taylor & Linforth, 2010).

Overall comparison of all three technologies mentioned above was compared in much more detail by Biasioli et al. (2011b) with examples of applications for each technology. These instrumental results could be correlated to appropriate sensory evaluations to help better understand the perception of flavour. For example, Shojaei et al. (2006a) carried out simultaneous sensory and instrumental studies with about 90 people, which showed that the in-vivo results during the consumption of milk (proximal stimulus) related better to sensory perception than the headspace analysis (distal stimulus). Further application of their work on reformulation will be mentioned in Chapter 4.

### **1.3 FLAVOUR STABILITY**

#### **1.3.1 Flavouring Definition & Classification**

The flavour of the food products can be improved and modified with the use of flavourings. The definition of 'flavouring' depends on the regions of which authorities issue their guidelines, taking the US and EU regulation for instance. a) The United States as a member of Flavor and Extract Manufacturers Association (FEMA.) and it applies the Code of Practice of the International Organization of the Flavor Industry (IOFI, 2006), which defines flavourings as 'products that are added to food to impart, modify, or enhance food additives, with the exception of sweet, sour, or salty taste.' b) The EU Commission have established new flavour regulation (EC/1334/2008) with two clear points as flavouring definition: 'i) not intended to be consumed as such, which are added to food in order to impart or modify odour and taste; ii) made or consisting of the following categories:

flavouring substances, flavouring preparations, thermal process flavourings, smoke flavourings, flavour precursors or other flavourings or mixtures thereof’.

Although the above definitions differ in some aspects, the terms of ‘flavourings’ in the flavour industry are generally regarded as the complex compositions of edible chemicals and extracts that alter the flavour of the food product. Furthermore, the perception of flavour is a property of flavourings, for example, a typical strawberry flavouring in a candy will be perceived as a strawberry flavour.

Flavourings could cover a wide range of categories, but the key areas of interest in this thesis are flavouring substances, which are chemically defined substances with flavouring properties. These substances could be either formed by chemical synthesis, or obtained from materials of plant or animal origin. Natural flavouring substances are naturally present and have been identified in nature that are obtained from vegetable, animal or microbiological origin either in the raw state or after a traditional food preparation process for human consumption. Regarding to this study, most aroma compounds are chemically synthesized because their composition can be precisely controlled.

Despite the source of the flavouring substances, they can be classified through their functional groups such as acids, esters, alcohols, aldehydes and ketones, etc. These functional groups have some significant effect on their aroma profile and numerous attempts have been made to provide a theory to explain structure-activity relationships (Lipkowitz, 1989). Maurice (2002) concluded that the most important factors affecting this relationship seemed to be overall shape and size of the molecule, certain chemical properties (e.g. polarity, nature of functional groups) and physical properties (e.g. volatility and solubility).

Flavourings are commonly divided into classes based on the physical states, i.e., liquid, emulsions, powder or paste. Liquid flavourings are the major products sold by Aromco, and they are the only type used in our studies. Typical liquid flavourings are made by blending the required flavouring substances in the desired concentration with particular food grade solvents, which are discussed in the following section.

### 1.3.2 Flavour Solvent

Selecting an appropriate solvent for a liquid flavouring is based on its ability to dissolve the required flavouring compounds and its solubility in the applied food products. For example, flavourings for beverage need to use water soluble solvent like ethanol and propylene glycol, whereas bakery products or salad dressing prefer more oil soluble solvents like triacetin or vegetable oil. Furthermore, use of the flavour solvent should also avoid an adverse effect on the product properties. de Roos (2007) suggested that propylene glycol works as a plasticiser and makes the hard candy sticky so it should be replaced in the flavourings for hard candies. Another well-known example is triacetin being widely used in the chewing gum flavouring due to the gum formulated with triacetin is softer than if formulated with propylene glycol (Potineni, 2008).

According to the strict selection criteria, the number of approved solvents available for food flavouring is limited. The commonly used solvents in the global flavouring industry are propylene glycol, ethanol, glycerol, and triacetin. However, some solvents have limited application due to technical and cultural reasons: ethanol is not permitted in Muslim countries and its transportation implies higher cost; glycerol is a poor solvent for many flavouring compounds. This leaves

propylene glycol (PG) and triacetin (TA) being the widely accepted flavouring solvents as the main areas of interest in this study. The chemical structure of PG and TA is shown in Figure 1.5.

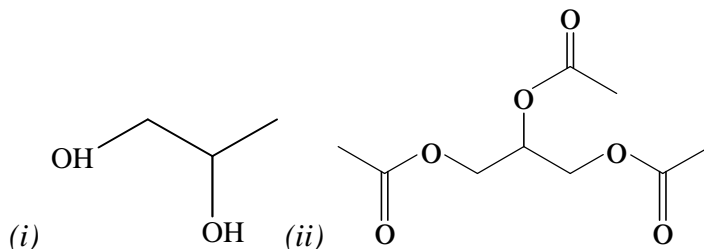


Figure 1.5 Chemical structure of i) propylene glycol and ii) triacetin

#### 1.3.2.1 Propylene glycol (PG)

Propylene glycol (1,2-propanediol) is a colourless and slightly viscous liquid with a faintly sweet taste (Acrctander, 1969). Since it is miscible with water, alcohol and most flavour compounds, PG being relatively inexpensive becomes the most extensively used flavour solvent.

From the chemical structure of PG shown in Figure 1.5 (i), two hydroxyl groups can be very reactive with either other flavour compounds in the flavouring or other major components in the food products. Therefore, PG can result in the formation of new compounds due to this reactivity.

The reaction of PG with aldehydes and ketones to form the corresponding acetals and ketals, has been widely reported (Heydanek & Min, 1976). This reaction can be reversible in aqueous acidic media where acetals are quickly hydrolysed to regenerate the original aldehydes (de Roos, 2007). Sharma et al. (1998) considered the acetal formation as an advantage because it protects the aldehydes against oxidation and polymerisation in the flavouring; while Baines and Knights (2005) mentioned in some cases the acetals may be insoluble in propylene glycol forming two layers in the flavour.



In addition to PG-acetal formation, Elmore et al. (2011) also summarised another two reaction pathways during storage of PG flavourings, i.e., i) reaction between PG and organic acids commonly used in flavourings (e.g. acetic and butyric acid) to form both monoesters and diesters; ii) transesterify with lactones to give dihydroxy esters.

#### 1.3.2.2 Triacetin (TA)

Compared to propylene glycol, triacetin (1,2,3-propanetriol triacetate) is a colourless, slightly viscous liquid with a very faint ethereal-fruit odour (Acrctander, 1969). As indicated in Figure 1.5 ii), TA is less polar than PG and basically more oil soluble. It also has good solvent properties with a higher boiling point (260 °C) than PG (188 °C), as experimental values indicated by EPIsuite<sup>TM</sup>.

Since TA does not react with aldehydes, it can be used in flavouring when the use of PG is restricted or avoiding acetal formation becomes vital. TA as an ester may transesterify with other esters, or with alcohols to release acetic acid (Winkel, 2005). That may explain why Baines and Knights (2005) describes triacetin with ‘a mild sweet taste at level less than 500 pm but above is bitter and may have an acetic acid character depending on the extent of decomposition’.

On the other hand, TA had found another possible function by Choi et al. (2009) who incorporated TA into oil-in-water emulsions to alter the stability of citral to chemical degradation. Their results indicated that triacetin may improve the chemical stability of citral in beverage emulsions. However, they also noted that temperature fluctuations during storage or the presence of other additives may affect the rate of citral degradation, so they added ‘further studies are needed to

elucidate the impact of other factors on citral stability in model beverage emulsions’.

Nevertheless, the work on the impact of solvent choice on food product consumer quality and perceived preference over ageing is limited, especially comparing the effect of these two solvents (PG and TA) on the flavour performance within food products. Therefore, more comprehensive studies about solvent effect on flavour stability of biscuits during processing and shelf-life test were carried out in this study (Chapter 5, 6 and 7).

### 1.3.3 Factors Affecting Flavour Stability in Food Products

The evidence of flavour instability could be the disappearance of essential molecules and the appearance of other components with high flavour impact (Grab, 1994). The stability of the flavouring itself and its stability in the food need to be distinguished, so the nature of flavour compounds in flavouring itself is discussed in the Section 1.3.3.1, while the possible flavour changes during food processing and product storage are discussed in Section 1.3.3.2 and 1.3.3.3 respectively.

#### *1.3.3.1 Factors for flavouring compounds*

Flavouring itself is a complex composition of natural and synthetic flavouring substances, and its quality is characterised by the balanced quantity of relevant flavouring substances. Any change in this balance could change the perceived quality and the flavour profile. Flavouring compounds are labile and their stability varies according to different functional groups. Aldehydes are one of the most reactive compounds, which are particularly susceptible to oxidation to acids.

Whilst flavourings should have a shelf-life of at least a few months, solving flavour stability problems depends on its physical state (liquid or solid). In the liquid flavouring system, flavour compounds can react not only with other flavour compounds, but also with solvent. In particular, the interactions between flavour solvent (PG or TA) and certain flavour compounds have been reviewed in the previous section (1.3.2.1 or 1.3.2.2). Specific flavour compounds will react differently with individual solvents; and there is evidence of interactions between propylene glycol and aldehydes, such as cinnamaldehyde and vanillin could form their respective propylene glycol acetals (Potineni & Peterson, 2008; Elmore et al., 2011).

Possible reaction of flavour compounds through degradation, oxidation and rearrangement, illustrated the challenges when handling flavour stability issues. When dealing with flavourings, small changes of the quantity and the structure of ingredients may have a strong influence to the perceived quality of the flavoured food. The flavour intensity is gradually reduced with time, and the desirable flavour profile may change directly to be unbalanced and even unacceptable for the final product after food manufacturing.

#### *1.3.3.2 Factors for food processing*

Aroma compounds could be either generated or lost during food processing. There are two fundamental causes of flavour instability during food processing: i) chemical instability, where the concentrations of the flavour compound decrease due to decomposition in the product or reaction with food components; ii) physical instability, where the flavour compounds remain intact but disappear

from the product by volatilisation, migration, absorption and adsorption effects in complicated food matrices, or other physical processes.

Take baking for example: a large number of complex chemical reactions and physical processes lead to the generation of the biscuits final flavour. The key chemical reactions like the Maillard reaction and caramelisation (Ait Ameer et al., 2008) and the physical processes like volatilisation, physical binding and physical entrapment (de Roos, 2006) may all occur during baking. The impact of major components (flour, sugars and fats) on the biscuit dough system was reviewed by Chevallier et al. (2000b), this illustrated several complex phenomena during dough preparation and baking, such as protein denaturation, loss of starch granular structure and fat melting. All these transformations lead to the formation of final biscuit structure, demonstrated by Chevallier et al. (2000a) as a cellular solid with a thin coloured surface and a porous inner structure where proteins aggregate and starch granules remain almost intact in biscuits.

The resulting food structure or microstructure could have a significant impact on its texture (Pareyt et al., 2009), and hence the aroma release during eating (Burseg et al., 2007), thereby influence the related sensory perception such as crispness and hardness of cookies (Booth et al., 2003) or freshness of biscuits (Heenan et al., 2009). Additionally, the spatial geometry and homogeneity of the food matrix will also have a marked impact on the flavour release and stability (Druaux & Voilley, 1997). For example, the effect of viscosity or presence of physical barrier layers (de Roos, 2003) could lead to differences in limiting factors e.g. diffusion rates, permeability, porosity, and tortuosity.

The major factors that can affect flavour stability during food processing are process temperature and time, product moisture content, pH of the food matrix and other product variables. In our study, all the processing conditions were kept as similar as possible to minimise the variation of biscuits during production.

#### *1.3.3.3 Factors for product storage*

During product storage, shelf-life is an important concept, which is defined as the time during which the food product will: i) remain safe; ii) be certain to retain desired sensory, chemical, physical and micro-biological characteristics; iii) comply with any label declaration of nutritional data when stored under the recommended conditions (Kilcast & Subramaniam, 2000). The factors that influence shelf-life can be categorised into intrinsic and extrinsic factors. Intrinsic factors are the properties of the final product, like water activity, pH values that are determined by its raw material type and quality, and product formulation and structure. Extrinsic factors are linked to storage conditions of the final products, such as storage temperature, time, relative humidity, light and packaging.

The packaging material may interact with the flavour constituents of the food, which could cause the loss of desirable food flavour and absorption of undesirable off-flavour from components of the packaging (Sajilata et al., 2007). The interactions of aroma compounds between ingredients, packaging and atmosphere could cause either physical instability (by migration, volatilisation, absorption and adsorption) or chemical instability (by oxidation, photo-oxidation and fat autoxidation).

A number of processes that limit shelf-life are classified as microbiological, chemical, physical and temperature related. The growth of spoilage organisms is

often readily identified by sensory changes like visual mould growth, generation of off-odours and flavour or texture changes. In low water content food like biscuits, it is normally a minor concern. Physical deteriorative changes due to moisture migration are the major cause, especially for biscuits that lose their crispness through moisture uptake. Many chemical deteriorative changes occur from reactions between food components with external species, such as oxygen. In fat-containing foods like biscuits, rancidity development can occur via different mechanisms, e.g. lipolytic/hydrolytic reactions, oxidative reactions and flavour reversion reactions. Deterioration can occur at both elevated and depressed temperatures. In terms of temperature, it is known that increased temperatures will accelerate many ageing process.

Consequently, the stability of flavourings in products does not only depend on the nature of the flavouring compounds and solvent but also on the product composition, food processing, packaging and storage conditions.

To sum up, this chapter has provided the background to some key factors that influence flavour release from food and flavour stability in food products, which should be taken into account during flavour reformulation and stability studies. Further considerations to these two key areas are given in Part II and Part III.

## **PART II FLAVOUR REFORMULATION**

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### **2 INTRODUCTION**

#### **2.1 FLAVOUR REFORMULATION APPROACHES**

As many factors affect flavour release from foods (see Section 1.3.3), it is very challenging for the flavourists to produce the same flavour perception across different food matrices. Indeed, flavour houses may have ten to a hundred different flavour formulations of one typical flavour type (e.g. strawberry) to cope with many different matrices. Instead of using traditional process of reformulating flavours through trial and error, some novel approaches based on either instrumental analysis or modelling prediction have been used in a fundamental and scientific way during this study.

##### **2.1.1 Instrumental Analysis**

Taylor (1996) considered that for the same flavour experience to be perceived from different food matrices, the same profile of flavour compounds must be received at the flavour receptors in the nose and with the same timing. With the development of the APCI-MS technique, in-vivo measurement results have provided information about the intensity and timing of aroma release from some aroma components.

Hollowood et al. (2000) demonstrated that the concentration of volatile components released during eating measured by APCI was correlated with perceived quality and intensity of the flavour of a food. However, Hort and Hollowood (2004) then found that the perception of the banana flavour with controlled amounts of isoamyl acetate was driven by the sucrose concentration.

Taste-aroma interactions have been reported from several studies, such as Davidson et al. (1999) which illustrated that the perception of mint-flavour followed sucrose release rather than menthone release; Dalton et al. (2000) demonstrated an aroma-taste-interaction when benzaldehyde and saccharin were used as aroma stimuli and taste stimuli respectively.

Nevertheless, when the taste and trigeminal stimuli are similar during eating two food systems, the aroma release difference is assumed to be related directly to a sensory perception difference. This is the proposed hypothesis to use instrumental analysis as a novel flavour reformulation approach. If the aroma released during the eating of the two food systems with the same flavouring were measured by APCI-MS, comparing the aroma release profile between two systems for each compound would determine which compounds in the target flavouring were affected and by how much. Reformulating flavour becomes feasible using the ratio of the maximum breath concentration ( $I_{max}$  value) measured for target and investigated samples.

APCI-MS analysis has been applied widely to measure aroma release differences during consumption of foods with different fat contents, such as yoghurts (Brauss et al., 1999b), biscuits (Brauss et al., 1999a), emulsions (Doyen et al., 2001) and milk samples (Shojaei et al., 2006b). Various food applications have been studied, and all highlighted differences in flavour delivery from samples with different fat content, and the impact of the hydrophobic properties of the aroma compounds on release. As a result, this study investigated the effect of lipid content in food and hydrophobicity of the aroma compounds as two main factors for flavour reformulation regardless of using instrumental analysis or model prediction.



This instrumental approach for flavour reformulation was proved to be successful by Shojaei et al. (2006a). The group manipulated a fruit flavour aroma (ethyl hexanoate) in low fat milk to have similar delivery intensity in regular fat milk was perceived the same. However, they only dealt with simple milk systems using a single aroma compound, whereas real commercial flavourings are usually a much more complex mixture involving a wider range of compounds. Therefore, one of the purposes for this study was to evaluate the application on this lab-based technique to commercial flavourings added into real food systems.

### 2.1.2 Model Prediction

Aroma compounds have very different properties due to their chemical structure, and their interactions with food matrices to different extents. Equations can be constructed with parameters that describe the attribute of the system (flavour molecules, matrix and the phases surrounding them) and the way these components interact to influence flavour release. Therefore, modelling flavour release from these equations to predict flavour perception is an attractive proposition and has received attention from several research groups using three different approaches: theoretical models by Harrison and Hills' Group (Section 2.1.2.1), semi-empirical models by de Roos' Group (Section 2.1.2.2), and empirical models by Taylor and Linforth's Group (Section 2.1.2.3).

#### 2.1.2.1 *Theoretical models (Harrison & Hills' Group)*

Mechanistic models for flavour release were proposed by Harrison and Hills (1997). The basic models for mass transport were adapted for the special conditions that apply to a variety of food materials from a boiled sweet (Hills & Harrison, 1995), gelatine gels (Harrison & Hills, 1996), emulsion (Harrison et al.,

1997) and liquids where aroma binding occurred (Harrison & Hills, 1997). These models were comprehensive and covered key release mechanisms like saliva-product partition and mass transport of aroma across the liquid-gas interface, but validation of these models using experimental data has been limited because determining suitable values for the key parameters (like the diffusion coefficient) could be very difficult.

Linforth (2010) commented that the advantage of these models is that they are not initially dependent on available experimental data, which are only required for model validation. The disadvantage of these models is that they can be created and tested for simple systems but are difficult to work with in complex situations because too many variables can make a solution impossible. For example, Harrison (2000) has described the model of flavour release from chewing gum during eating with 16 variables in total. The values of all the variables in their models were determined by iterative fitting individually, and such processes are difficult and unreliable.

#### *2.1.2.2 Semi-empirical models (de Roos' Group)*

Unlike the Harrison and Hills models, which are based on an understanding of the physical processes involved in the mouth during eating, de Roos and Wolswinkel (1994) developed a model based on non-equilibrium partition. To overcome a lack of information on the mass transfer properties at the saliva-air interface, they obtained experimental data and developed a semi-empirical relationship that allowed them to produce predictive models. The predicted values from these models were found to correlate well with the observed behaviour from foods. This approach has apparently proven successful, not only in simple systems, but also in

real foods (de Roos & Graf, 1995; de Roos, 1999). The different models for flavour release from liquid and solid products have been reviewed by de Roos (2000).

The models developed by de Roos group combine the theory with a degree of empiricism, so they are more usable and practical than pure theoretical models developed by Hills group. However, the limitation of this approach is that these models only apply for the conditions pertaining to the experimental conditions and each new formulation of a product and each different food type would require a new model to be developed.

#### *2.1.2.3 Empirical QSPR models (Taylor & Linforth's Group)*

An alternative approach is to develop empirical models using Quantitative Structure Property Relationship (QSPR) modelling. This approach assumes the behaviour observed experimentally is a result of the different physicochemical properties of the compounds exhibiting the behaviour. Linforth (2010) described the process to establish the empirical models. Firstly, experimental data (such as aroma compounds in-vivo concentrations) are collected to determine the behaviour of a range of flavour compounds in a range of food matrices. Then the model is constructed with sufficient components to describe the variation in the data. This type of model may contain some of the same parameters used in the theoretical approach, or they may contain parameters that numericise the differences that occur in the range of the system studies (e.g. differences in Log P values).

Taylor and Linforth (2001) explained this QSPR modelling approach to predict dynamic flavour release from certain food systems. Friel et al. (2000) applied this

method to describe and predict the headspace concentration of volatile compounds above solutions containing sucrose. Carey et al. (2002) predicted the partition behaviour of volatile aroma compounds from a cloud emulsion. Kant et al. (2003) using QSPR, identified two key physicochemical properties (Log P and a molecular charge descriptor) being the factors determining flavour retention in dilute starch solutions.

To sum up, the empirical QSPR modelling is data driven and derived statistically based on correlation and regression. Best descriptors are selected to build the model and make accurate predictions, but it must be tested to ensure the reliability of the model's predictions. The model is validated by comparing the predicted values with the data obtained experimentally under the same conditions used for the prediction, and the quality of the correlation between these two sets of values is a good indicator of the validation of the model. Regarding to our study, a QSPR model that can predict the effect of fat on flavour release during mastication was developed and validated in real products.

## **2.2 RESEARCH OBJECTIVES**

The aim of the flavour reformulation study was to evaluate both instrumental and modelling approaches and their relative merits.

### **2.2.1 Objectives for instrumental approach to flavour reformulation**

(case study: confectionery products)

- (1) To define the variations related to APCI-MS in-vivo analysis of confectionery products.

Confectionery products are more complex food systems than the milk samples previously used for flavour reformulation (Shojaei et al., 2006b). It can be challenging to apply APCI-MS in-vivo analysis for confectionery products because large variations might be gathered from instrument variation during measurement, flavour content variation during confectionery manufacturing, and panellist variation during chewing and swallowing these candies. Thus, variations from these three sources need to be defined initially.

- (2) To evaluate the feasibility of applying in-vivo analysis by APCI-MS to reformulate real commercial flavourings.

Reformulating complex commercial flavouring is also challenging as previous research only measured the change in a single aroma compound by APCI-MS in-vivo analysis (Shojaei et al., 2006b). In this study, the challenge was greater, as a commercial strawberry flavouring that consisted of nine aroma compounds at various concentrations was selected. The challenge of the method and ability of APCI-MS to detect the nine compounds, and at the concentrations used, was tested.

- (3) To reformulate flavourings for chewy candy to achieve the same aroma release as pectin jelly via APCI-MS in-vivo analysis.

In the confectionery industry, pectin jelly as a fat-free soft matrix is normally considered as standard with good flavour release; whilst chewy candy, with 8% fat and a hard matrix, has much poorer release. When applying the same flavouring to a different matrix, some level of reformulation is usually required. Instead of using trial and error as the traditional approach, APCI-MS was used to measure the in-vivo release ratio between pectin jelly and chewy candy for every aroma compound. The strawberry flavouring was then reformulated using these ratios.

- (4) To validate the instrumental reformulation approach applied to chewy candy by comparing with pectin jelly via sensory analysis.

Ultimately, it is necessary to assess consumer perception of chewy candy with the reformulated strawberry flavouring by comparing it with the standard pectin jelly's flavour during sensory analysis. If there is no significant flavour difference between these two products ( $p > 0.05$ ) perceived by consumers, the instrumental reformulation approach is valid.

## 2.2.2 Objectives for modelling approach to flavour reformulation

(case study: yoghurt products)

- (1) To demonstrate the process of modelling reformulation approach in yoghurt products with different fat levels.

A general reformulation model was developed by Linforth et al. (2010) based on fat content in food matrices and compound hydrophobicity (Log P). Once these values are known, the model equation can be used to calculate the predicted in-vivo release differences between any two products, which can then be used for

flavour reformulation. For this study, yoghurt products at different fat levels with an experimental flavouring were selected as case study to illustrate this modelling reformulation process.

- (2) To reformulate flavourings for low fat yoghurt to deliver the same profile as its high fat version through predictive modelling.

Due to concerns about dietary intake and health, low fat food alternatives are preferred over their original high fat versions. However, removing fat in yoghurt products causes imbalanced flavour profiles when the same flavourings are applied as in the standard high fat version. Flavourings in low fat yoghurts are required reformulation so they deliver the appropriate aroma profile. Instead of actually measuring their in-vivo release differences between the selected two yoghurt products, the flavouring can be reformulated for the low fat yoghurt based on the values calculated by the model equation. Using these model's predicted values, the reformulated flavourings in low fat yoghurt are not expected to be significantly different to the high fat version ( $p > 0.05$ ).

- (3) To validate the modelling reformulation approach via instrumental measurement using APCI-MS in-vivo analysis.

To determine the precision of model's predictions, actual measurements of aroma release were made. If there is good correlation ( $r > 0.90$ ) between these predicted values and the actual in-vivo release ratios measured by APCI-MS for the original low fat and high fat yoghurt, the model gives a good prediction for what happened during in-vivo analysis. Additional APCI-MS analysis can be applied to measure the release from low fat yoghurt with reformulated flavouring from the model's prediction. If there is no significant difference ( $p > 0.05$ ) between the release of the reformulated low fat yoghurt and the standard release from the original high

fat yoghurt, the modelling approach is further confirmed as a valid method for flavour reformulation.

- (4) To validate the modelling reformulation approach applied to yoghurt products via sensory analysis.

Finally, sensory validation of the modelling approach is required by comparing the flavour of the two yoghurts. However, reducing fat level in yoghurt products also induces changes in texture and mouth-feel, so the perception might be affected even if the aroma release is matched between these products. Additional sensory analysis can compare both the original and reformulated flavourings in low fat yoghurt, and if consumers could not differentiate them ( $p > 0.05$ ), the limitations of this model's application need to be defined.



## PART II FLAVOUR REFORMULATION

### 3 MATERIALS & METHODS

To meet the objectives outlined in the previous section, an experimental flavouring and several commercial flavourings were used to assess the feasibility of reformulating flavourings through both instrumental and modelling approaches. The methodological approach for the flavour reformulation study is summarised in Figure 3.1.

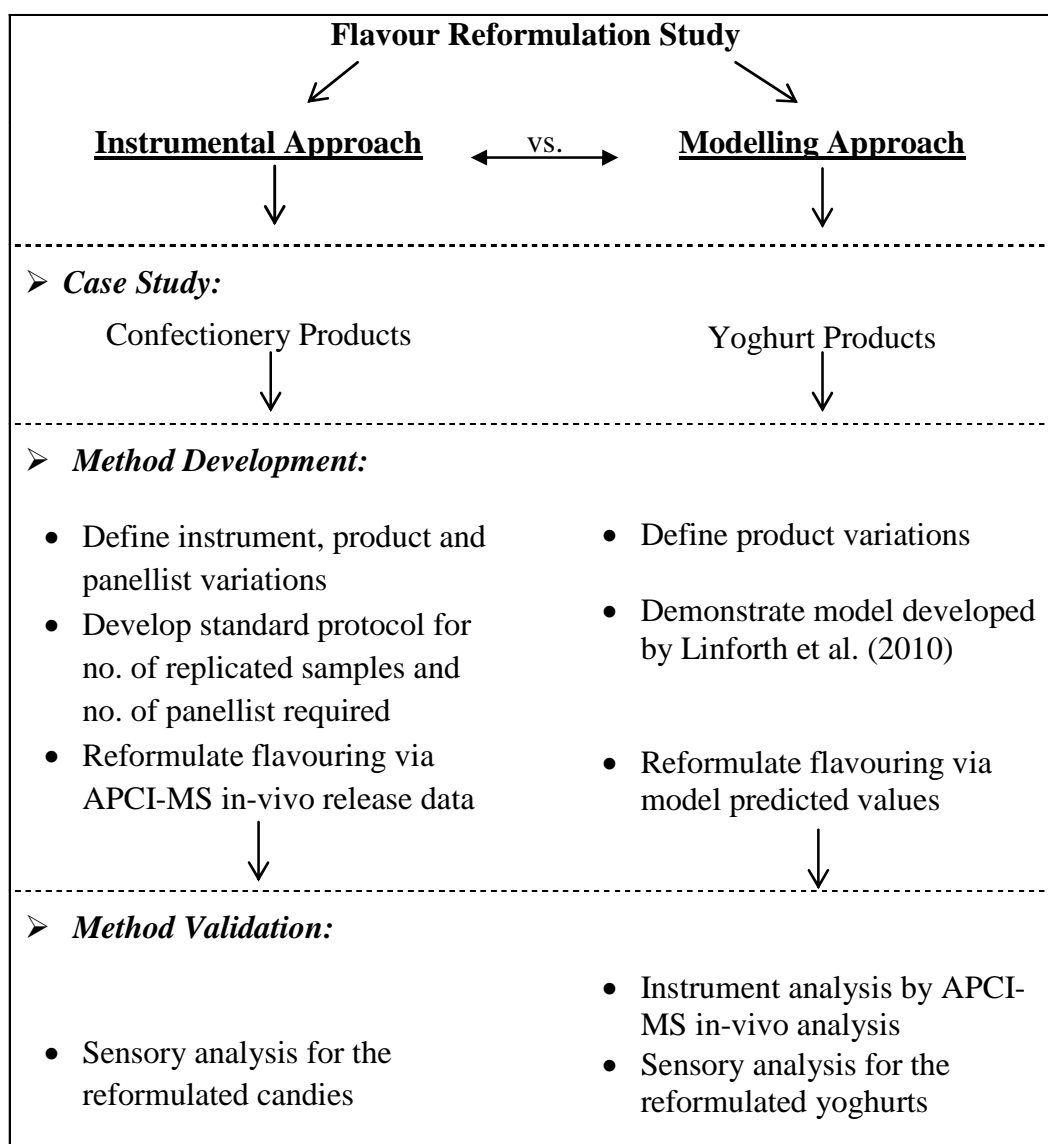


Figure 3.1 Schematic diagram of methodology applied in flavour reformulation study through instrumental and modelling approach

Confectionery products were used as a case study to illustrate the instrumental reformulation approach. Since real flavourings and real food systems were the materials used in the experiments, it was necessary to identify the major sources of variation involved during reformulation studies. Therefore, the tests for variation analysis were carried out prior to the reformulation studies and a standard method was developed for headspace and in-vivo analysis by APCI-MS for all subsequent reformulation studies. A commercial strawberry flavouring was reformulated for chewy candy comparing its in-vivo release ratio with pectin jelly. The results of reformulated flavouring for chewy candy were then validated by sensory analysis.

For the modelling approach, flavoured yoghurt products with low fat and high fat versions were used in the case study to demonstrate the modelling reformulation. A mathematical model was applied to reformulate flavourings for low fat yoghurt to achieve the same flavour release as high fat yoghurt. The predicted values from model equation were validated by the release results from APCI-MS in-vivo analysis. Sensory tests were also applied as another validation method to assess consumer perception for the original and reformulated flavourings in the selected yoghurt products.

### **3.1 FLAVOURINGS**

#### **3.1.1 KTP4 Experimental Flavouring**

The selection of compounds for KTP4 experimental flavouring was based on four criteria: i) they should be commercially available aroma compounds; ii) they should be volatiles that can be detectable by APCI-MS; iii) they should vary in hydrophobicity and cover a broad range of Log P values; iv) they should vary in

functional groups that include aldehydes, esters, alcohols and ketones. Previous illustration with the range of compound hydrophobicity and volatility (Chapter 1, Figure 1.3) was used as reference, and the following six compounds that fulfilled all the requirements were chosen (Table 3.1).

Pyrazine (Aldrich; Dorset, UK), 3-methyl butanol, ethyl butyrate, 2-nonanone, para-cymene, ethyl nonanoate and propylene glycol were all supplied by Aromco Ltd (Nuthampstead, UK) at purity levels > 95%.

*Table 3.1 Composition of an experimental flavouring (KTP4) and APCI ions used to monitor their release at cone voltage of 18 V*

<b>Compound</b>	<b>Log P</b>	<b>Molecular Mass (Da)</b>	<b>Formulation (g)</b>	<b>APCI ion (<i>m/z</i>)</b>
pyrazine	-0.06	80	0.50	81
3-methyl butanol	1.26	88	0.25	71
ethyl butyrate	1.85	116	0.05	117
2-nonanone	2.71	142	0.25	143
<i>p</i> -cymene	4.00	134	0.25	134
ethyl nonanoate	4.30	186	0.25	187
propylene glycol	-	-	8.45	-

The Log P value was estimated by KOWWIN<sup>TM</sup> programme from EPIsuite<sup>TM</sup>, which was developed by the US Environmental Protection Agency's Office of Pollution Prevention Toxics and Syracuse Research Corporation with free download available (<http://www.epa.gov/oppt/exposure/pubs/episuite.htm>). This EPIsuite<sup>TM</sup> also provides other physicochemical parameters (e.g., boiling point, melting point, vapour pressure, air-water partition coefficient, etc.). So any relevant parameters involved in this thesis were estimated by this software.

This KTP4 experimental flavouring was applied in the confectionery products for variation analysis and in the yoghurt products for modelling reformulation demonstration and instrumental validation.

### 3.1.2 Strawberry Flavouring

A commercial strawberry flavouring including nine compounds (Table 3.2) was used to demonstrate instrumental reformulation approach in the confectionery products and to validate modelling reformulation approach in yoghurt products for sensory analysis. All chemicals were supplied by Aromco Ltd (Nuthampstead, UK) and they were mixed in propylene glycol at confidential levels recommended by Aromco. Due to the confidentiality, the concentration of every compound could not be disclosed in this thesis.

*Table 3.2 Nine compounds in a strawberry flavouring demonstrated by their respective Log P values and APCI ions used at cone voltage of 18 V*

<b>Compound</b>	<b>Log P</b>	<b>Molecular Mass (Da)</b>	<b>APCI ion (m/z)</b>
diacetyl	-1.34	86	87
furaneol	0.82	128	129
butyric acid	1.04	88	89
cis-3-hexanol	1.61	100	83
ethyl butyrate	1.85	116	117
methyl cinnamate	2.36	162	163
$\gamma$ -decalactone	2.57	170	171
ethyl hexanoate	2.83	144	145
hedione	2.98	226	227

### 3.1.3 Other Commercial Flavourings for modelling reformulation

Besides strawberry flavouring, three other commercial flavourings (blackcurrant, banana and coffee flavourings) were supplied by Aromco. These four flavourings were reformulated by the model approach, and all the original flavourings and their reformulated flavourings were applied into low fat yoghurt for sensory validation.

## 3.2 INSTRUMENTAL APPROACH

### 3.2.1 Confectionery Products Preparation

Two types of confectionery products were chosen to demonstrate the effect of fat on aroma release and the reformulation approach through instrumental analysis. Pectin jelly as a fat-free soft matrix is normally used as standard flavour profile in the confectionery industry as it is considered to have a good flavour release. Whereas chewy candy with 8-9% fat is known to have much poorer release if the same amount of flavouring was applied. Therefore, the flavouring for chewy candy normally needs to be reformulated to the target aroma profile of pectin jelly.

#### 3.2.1.1 *Flavouring Addition*

The KTP4 flavouring was added at the same concentration (0.2%) to pectin jelly and chewy candy to check the product variation. The KTP4 flavoured candies were used to develop method for instrument measurement.

According to the dosage suggested by Aromco, the strawberry flavouring was added at 0.2% into pectin jelly and chewy candy, i.e., 1 g per 500 g candy mass. The reformulated strawberry flavouring through instrument approach was also added into chewy candy at 0.2%.

#### 3.2.1.2 *Pectin jelly manufacturing*

Pectin jelly (P) was made by a high ester pectin extracted from citrus peel and standardised by addition of sucrose (GENU® pectin 150 grade USA-SAG type D) supplied by CP Kelco Ltd (Grossenbrode, Germany). This pectin has a low setting temperature and is a common gelling agent used for confectionery products.

The pectin (11.25 g) was blended with 150 g sucrose (granulated sugar, Tate & Lyle Ltd, UK), added to 150 mL of boiled water (still water, Tesco Ltd, Perthshire, Scotland) and stirred until all clumps dissolved. The liquid was brought back to the boil and added with the remainder of the sucrose (232.50 g) and 150 g glucose syrup (42 °DE, Essential Ingredients Ltd, Kent, UK). The mixture was boiled until the temperature reached 109 °C, then removed from the heat to add 6.0 g citric acid solution (Cargill Ltd, Manchester, UK) prepared at 50% w/v solution. Finally, 1 g flavouring (0.2%) was added based on total yield of 500 g. The colouring Allura Red (Blends Ltd, Liverpool, UK) prepared at 50% w/v solution was added at 0.2% (1 g) into the candy mass.

Once the liquid mass was mixed well with the flavouring and colouring by wooden spoon for 1 min, it was quickly deposited into plastic moulds (each round pocket was 35 mm diameter and 5 mm thickness) to produce individual pieces. All sweets were left for 20 min to cool and set, then coated with fine sugar (Tate & Lyle Ltd, UK), wrapped and stored in air tight containers prior to consumption. Normally, 50 – 60 individual pieces of pectin jelly ( $5 \pm 0.5$  g) were produced from one standard batch.

#### *3.2.1.3 Chewy candy manufacturing*

Several ingredients used for chewy candy were from the same supplier as the pectin jelly, including sucrose, water, glucose syrup and citric acid. Additional ingredients for chewy candy were egg white power and hydrogenated palm kernel oil (HPKO) both supplied by Silbury Ltd (Warwick, UK).

Chewy candies were made by preparing a ‘Frappé’ consisting of egg white powder (20 g), water (80 g) and glucose syrup (320 g) by gradually whisking

(Hobart, Windsor, UK) for 10 min at 20 °C. A 'Premix' was made from glucose syrup (272 g), sucrose (184 g) and water (45 g) heated to 125 °C. The 'Frappé' (50 g) was added into hot 'Premix' by whisking for 1 min, and then combined with 45 g of melted HPKO followed by 3 min whisking. Based on a total yield of 500 g, 0.6% citric acid (3 g), 0.2% flavouring (1 g) and respective colouring - Allura Red (Blends Ltd, Liverpool, UK) were added and mixed by a metal beater (Hobart, Windsor, UK) for 2 min.

The hot mass was then layered onto a PTFE board to rest for 3 min, and then lifted by two plastic scrapers to form a rope shape by hands. After repeat stretching out and folding back for 5 min, the mass had a soft sheen appearance and was rolled to a thickness of 8mm. Individual candies were cut (15 x 15 mm), then wrapped and stored in airtight containers until use. One batch produced 60-70 pieces of chewy candy ( $5 \pm 0.5$  g).

All the ingredients used to make pectin jelly and chewy candy are summarised in Table 3.3.

Table 3.3 Pectin jelly and chewy candy composition in finished products. Values are (g/100 g product).

Ingredients	Matrix type	
	Pectin	Chewy
Sucrose	41	33
Glucose Syrup	38	56
Water	19	2.5
Pectin	2	
<b>Palm Oil</b>		<b>8.1</b>
Egg White Powder		0.4
	100	100
Flavouring	0.2	0.2
Colouring	0.2	0.2
Citric Acid	0.6	0.6

Since the same level of flavouring was added to both types of candies, the fat content at 8 % in chewy candy was considered as the major factor that reduced flavour quality comparing with the standard flavour profile of pectin jelly.

### 3.2.2 Variation Analysis Method

Three main sources of variation during flavour release analysis for two confectionery products were measured; i) instrument variation through APCI-MS measurement, ii) product variation within and between batches of candies, and iii) panellist release profile variation due to differences in oral processing between individuals.

#### 3.2.2.1 Instrument variation analysis

The instrument variation was evaluated through the static headspace analysis of the standard solutions of KTP4 flavouring. Dilutions of the KTP4 flavouring were made to produce standard solution containing 312 µg/L of pyrazine, 156 µg/L of 3-methyl butanol, 2-nonanone, *p*-cymene and ethyl nonanoate and 31 µg/L of ethyl butyrate. Four replicated samples of standard solution (200 mL) were placed



in screw capped bottles (500 mL) and allowed to equilibrate at 20 °C for 3 hours prior to the measurement. Three replicated measurements were taken by headspace analysis by APCI-MS (Platform II, Micromass, Manchester, UK) fitted with a MS Nose interface (Micromass) and operated at a cone voltage of 18V.

A portion of the headspace was sampled into the APCI source at flow rate of 6 mL/min through a small port in the bottle cap. Since the headspace volume in the flask was around 300 mL and sampled for a short time, there was no significant effect on headspace dilution and the APCI-MS traces showed a rapid rise in signal to a plateau value which was maintained for about 30 s until the bottle was resealed.

Data were collected in selected ion recording mode with a dwell time of 0.02 s monitoring ions with respective  $m/z$  value illustrated in Table 3.1. Masslynx 3.2 (Micromass, Manchester, UK) was used to determine the peak height of the maximum signal (i.e, the ion intensity) observed for every compound from every bottle at each measurement.

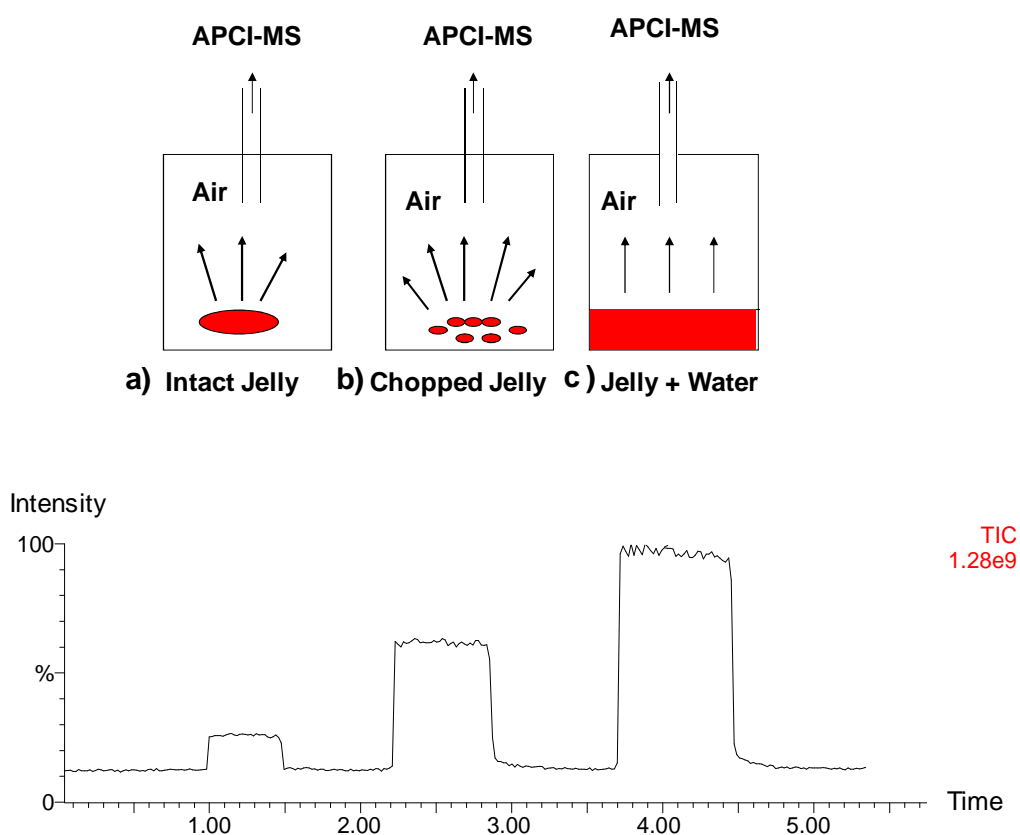
#### 3.2.2.2 *Product variation analysis*

##### *(A) Method development of headspace analysis for confectionery products*

Before measuring the product variation, it was essential to determine the best method for the headspace analysis of these candies. Three types of sample treatment methods were demonstrated by three pieces of strawberry flavoured pectin jelly. Each jelly (5g) was placed in the 500 ml glass bottle either intact (a) or chopped into pieces (b); the third piece was dissolved into 100 mL water (c). All the bottles were sealed with lids and after three hours equilibrium, the headspace above the sample was measured through a small port in the lid. The

same method of headspace analysis for all three types of sample preparation methods was carried out by APCI-MS (Platform II, Micromass, Manchester, UK) with the air flow of 6 mL/min.

Figure 3.2 shows the schematic demonstration of three sample treatments and respective chromatogram obtained during static APCI headspace analysis at each treatment under Full Scan Mode.

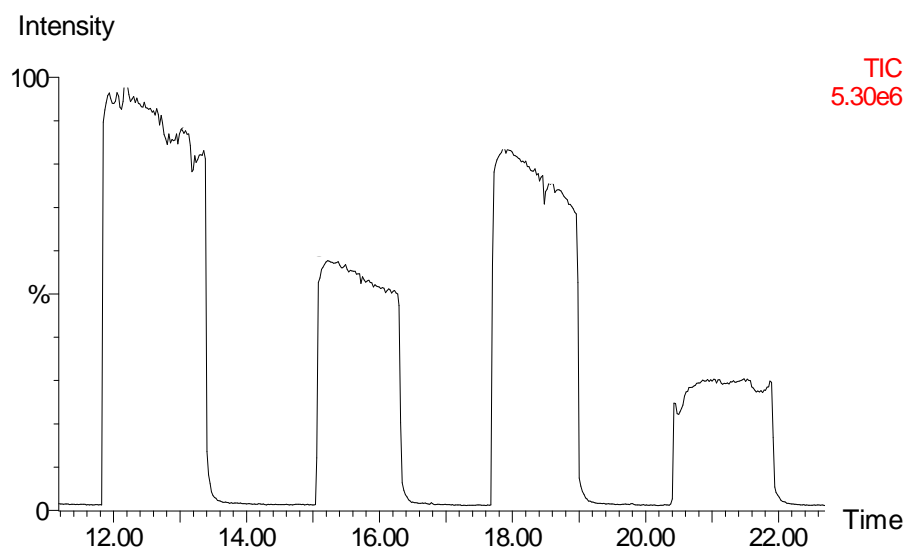


*Figure 3.2 Three sample treatment methods- a) intact jelly, b) chopped jelly and c) jelly dissolved in water with respective chromatograms by APCI-MS headspace analysis and the total ion current (TIC) by  $1.28 \times 10^9$  as the maximum ion intensity from three measurements taken at 1 min, 2.4 min and 3.8 min*

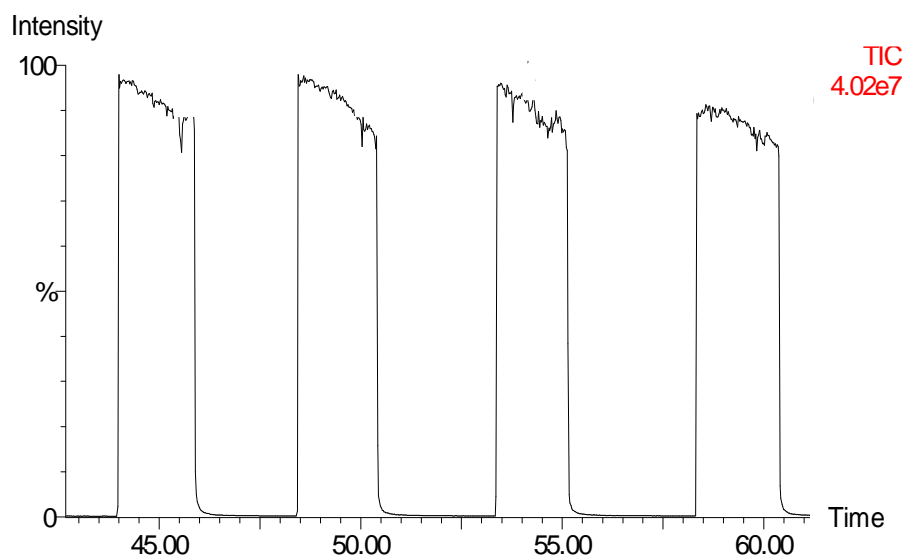
The results indicated that intact jelly (a) had the lowest TIC (total ion current) as compounds entrapped in the candy matrix were hardly released from intact jelly - only two ions were found for two out of nine compounds in the strawberry flavouring. As a result, the remaining two methods (b and c) with higher intensity were more practicable and represented more aroma compounds ions. Additional

four replicated samples were prepared for these two methods, and their chromatograms at the Selected Ion Recording Mode are shown in Figure 3.3.

i) Four **dry** chopped pectin jellies



ii) Four **wet** pectin jellies



*Figure 3.3 Chromatogram of i) four dry chopped pectin jellies; ii) four wet pectin jellies by APCI headspace analysis and TIC was 5.30 e6 and 4.02 e7 as the maximum ion intensity*

Figure 3.3 clearly shows that the measurement of the four replicate dry chopped jellies had larger variation in unit intensity, whilst the TIC measured for wet

jellies samples was higher and the intensity remained more consistent between four reps. In addition, wet jelly gives a comparable situation in mouth as saliva provides an aqueous environment. Therefore, dissolving candies into water was used as the standard headspace analysis method of confectionery products for this study.

*(B) Analysis of product variation*

Within-a-batch variation was assessed to check for potential variation from candy to candy, that is the distribution of flavouring within a batch of product. Batch to batch variation was assessed to check the additional factor of differences in flavour incorporation between batches of samples since there may be flavour loss or flavour incorporation differences. The headspace analysis was applied as a convenient and rapid method for measuring the intra- and inter-batch differences during candy manufacturing.

Five batches of pectin jelly samples and three batches of chewy candies were made on different days with 0.2% KTP4 flavourings. From these eight batches, three individual candies were randomly selected for analysis. From each candy, a 5 g portion was accurately weighed before dissolving it in 100 mL water. The dissolved solution was placed in a 500 mL glass bottle, and after equilibration the headspace in each bottle was measured by sampling headspace at 5.2 mL/min into an APCI-MS (Platform II, Micromass, Manchester, UK) fitted with a MS Nose interface (Micromass) and operating at a cone voltage of 18 V. The ion intensity was recorded for every compound from every candy.

### 3.2.2.3 *Panellist variation analysis*

#### *(A) Method development of in-vivo analysis*

With the development of aroma in-vivo release measurements by APCI-MS, the release patterns resulting from food consumption can be investigated based on in-vivo release profiles of panellists. The measurement could be taken either using their own way of eating (a free chewing style) or controlling the rate and time of chewing and swallowing (an imposed consumption).

Aprea et al. (2006) showed the free and imposed protocol influenced in-vivo aroma release from custard: subjects who swallowed relatively quickly released more flavour from the firmer custard than from the softer custard, whereas subjects who swallowed less rapidly released more flavour from the softer custard. However, the main disadvantage of an imposed protocol is that it modifies the mastication process, and in particular minimises its adaptation to the texture of the product. Therefore, the choice between a free or imposed consumption protocol must be made as a function of the objective of the study. The end users from our reformulation studies would be consumers who eat the products by their own style, so 'free-style' chewing needed to be applied in this study.

To extract data from in-vivo aroma release profile, the most widely used parameters are the area under the curve (AUC), maximum ion intensity (Imax) and the time to reach maximum intensity (Tmax) (Gierczynski et al., 2011). Previous reformulation work by Shojaei et al. (2006a) showed that the data could be better compared by calculating the ratio of the maximum breath intensity (Imax) between the two products for each panellist. So a similar method to compare Imax difference between panellist for every compound was applied in this study.

### *(B) Analysis of panellist variation*

Due to the potential impact that variation in oral physiology may have on flavour release and perception, the panellist variation was considered as a variable during the flavour release analysis in this study. Ten volunteers (denoted by the letters A, K, M, P, R, S, J, O, X and N) ate three replicates of the strawberry-flavoured pectin and chewy candies, known as 10 x 3 system. Another system involving four panellists (R, S, L, N) eating 9 replicates of the KTP4 flavoured pectin and chewy candies, was called 4 x 9 system. These two systems were used to study the panellist variations and to develop the standard protocol for instrumental reformulation approaches.

The APCI-MS was fitted with a MS Nose interface (Micromass) and was operated in selected ion mode with the cone voltage at 18 V. While panellists were eating the sample, their exhaled breath was sampled at 30 mL/min air flow rate from one of their nostrils. Panellists were asked to eat the candies in their own way but to try and be consistent in their eating pattern for each replicate.

Each panellist had 15-20 min rest periods between each sample during which they swilled their mouth with water and cleansed their palate with crackers. Prior to consuming a sample, the exhaled air from the nose of each volunteer was monitored to ensure that there was no carry-over of aroma from the previous experiment and that all compounds had returned to baseline levels. All the release experiments were carried out over a period of 4 h.

#### *3.2.2.4 Data analysis for variations studies*

To evaluate the results from the variation analysis, the average (AV), standard deviation (STD) and coefficient of variation (CV%,  $= AV \div STD \times 100$ ) were

calculated where appropriate. Regarding to the panellist variations calculated as CV% for every panellist, the paired t-test was applied to determine if there is a significant difference between CV% of pectin jelly and CV% of chewy candy ( $p < 0.05$ ).

### 3.2.3 Instrumental Reformulation Method

#### 3.2.3.1 Headspace analysis of individual flavour compounds

In order to check if every compound in the strawberry flavouring could be detected by APCI-MS, individual standard solutions were prepared for every compound. According to the lower detection limit for APCI-MS measurement, a concentration of 500 ppb was chosen as the gas concentration above every standard solution. The three major steps are shown below to make the standard solution, taking ethyl butyrate (MV = 116 g/mol) as an example included.

First of all, the concentration of gas ( $C_{\text{gas}}$ , mg/m<sup>3</sup>) was calculated based on 500 ppb detectable level. Since 1 mole gas of ethyl butyrate is 116 g (at standard atmosphere pressure 1 mole gas occupies 22.4 dm<sup>3</sup>), 1 dm<sup>3</sup> contains 5.18 g gas ( $116 \div 22.4$ ) and 1 ppm equals 5.18 mg/m<sup>3</sup> ( $10^{-6} \times 5.18 \times 10^6$  mg/m<sup>3</sup>). The gas concentration at 500 ppb was calculated as 2.59 mg/m<sup>3</sup> ( $0.5 \times 5.18$ ).

Secondly, using the estimated  $K_{\text{aw}}$  of ethyl butyrate as  $1.68 \times 10^{-2}$  (Unitless, EPIsuite<sup>TM</sup>), the concentration of solution ( $C_{\text{liquid}}$ ) was calculated as 0.154 mg/L, i.e., by rearrangement of  $K_{\text{aw}} = \frac{C_{\text{gas}}}{C_{\text{liquid}}} \cdot C_{\text{liquid}} = \frac{C_{\text{gas}}}{K_{\text{aw}}} = \frac{2.59}{1.68 \times 10^{-2}} = 154 \text{ mg/m}^3$ .

Finally, the volume ( $V$ , µl/L) required to make a standard solution was calculated as 0.176 µl/L based on the density of ethyl butyrate 0.879 kg/L or 0.879 mg/µl ( $V = \frac{m}{d} = \frac{0.154}{0.878}$ ).

Similarly these calculations were applied to other compounds, and the volume required (V) to prepare respective standard solution could be obtained. Table 3.4 summarises the estimated  $K_{aw}$  value and the calculated values of  $C_{gas}$  and  $C_{liquid}$ , and Volume for each compound.

*Table 3.4 Summary of calculated results to make the standard solution of every compound in strawberry flavouring*

<b>K<sub>aw</sub></b>	<b>Compound</b>	<b>C<sub>gas</sub> (mg/m<sup>3</sup>)</b>	<b>C<sub>liquid</sub> (mg/L )</b>	<b>Volume (µl/L)</b>
1.68×10 <sup>-02</sup>	ethyl butyrate	2.59	0.154	0.176
2.96×10 <sup>-02</sup>	ethyl hexanoate	3.22	0.109	0.124
2.30×10 <sup>-02</sup>	gamma-decalactone	3.80	0.165	0.168
5.47×10 <sup>-04</sup>	diacetyl	1.92	3.51	3.58
6.33×10 <sup>-04</sup>	cis-3-hexenol	2.23	3.523	4.16
6.01×10 <sup>-04</sup>	furaneol	2.86	4.75	4.75
1.69×10 <sup>-04</sup>	methyl cinnamate	3.62	21.39	21.39
2.40×10 <sup>-05</sup>	butyric acid	1.97	81.88	84.93
2.05×10 <sup>-05</sup>	hedione	5.05	246.10	246

The standard solution for each compound was prepared in distilled water (purified by Ondeo ‘Purite Select’) and then stored in 500 ml screw cap glass bottles. After 10 min to reach static equilibrium in the bottle, their headspace analysis was carried out by APCI-MS (Platform II, Micromass, Manchester, UK) with a sampling air flow of 6 mL/min. The operating cone voltage was 18 V with full scan mode in a range of 10 to 240 m/z.

### *3.2.3.2 In-vivo analysis during candy consumption*

During in-vivo analysis of candy consumption for the the reformulation study, five panellists participated to eat three replicates of the pectin and chewy candies with original strawberry flavouring added. Panellists were asked to eat the candies in their own way but to try to be consistent in their eating pattern. The aroma released from each panellist was collected by APCI-MS fitted with a MS Nose



interface (Micromass) at a sampling rate of 30 mL/min. It was operated in selected ion mode with the cone voltage set at 18 V. Each panellist had 15-20 min rest periods between each sample to cleanse their palate with water and crackers (Rakusens's 99% fat free, Rakusen's Limited, UK).

#### 3.2.3.3 *Data analysis for in-vivo results*

Shojaei et al. (2006a) pointed out that the inter-panellist variations due to different oral physiology could be minimised by using a ratio per panellist. Therefore, the approach used in this study was to determine the release ratios between the  $I_{max}$  values of pectin jelly (P) as standard, and  $I_{max}$  values of chewy candy (C), known as the P/C ratio for each compound by person. For example, the APCI-MS trace for a single panellist eating one pectin jelly and one chewy candy with six compounds in KTP4 flavouring is shown in Figure 3.4.

Figure 3.4 shows the release profiles and the maximum release intensity of hydrophobic compounds (ethyl nonanoate, 2-nonanone and cymene) was reduced substantially from chewy candy compared with pectin jelly, so their P/C ratios are more than 1.0. The  $I_{max}$  for more hydrophilic compounds (pyrazine and 3-methyl butanol) was higher from chewy candy than pectin jelly, so their P/C ratio are less than 1.0. While the trace for ethyl butyrate with intermediate hydrophobicity was in the middle of the two other groups of compounds, so its P/C ratio is around 1.0.

Similarly, the P/C ratio for each compound in strawberry flavouring would be calculated for every panellist. The average P/C ratio were calculated from five panellists for each compound, and its original concentration in the flavour formula divided by its average P/C ratio provides the adjusted concentration in the reformulated flavouring to be applied into chewy candy.

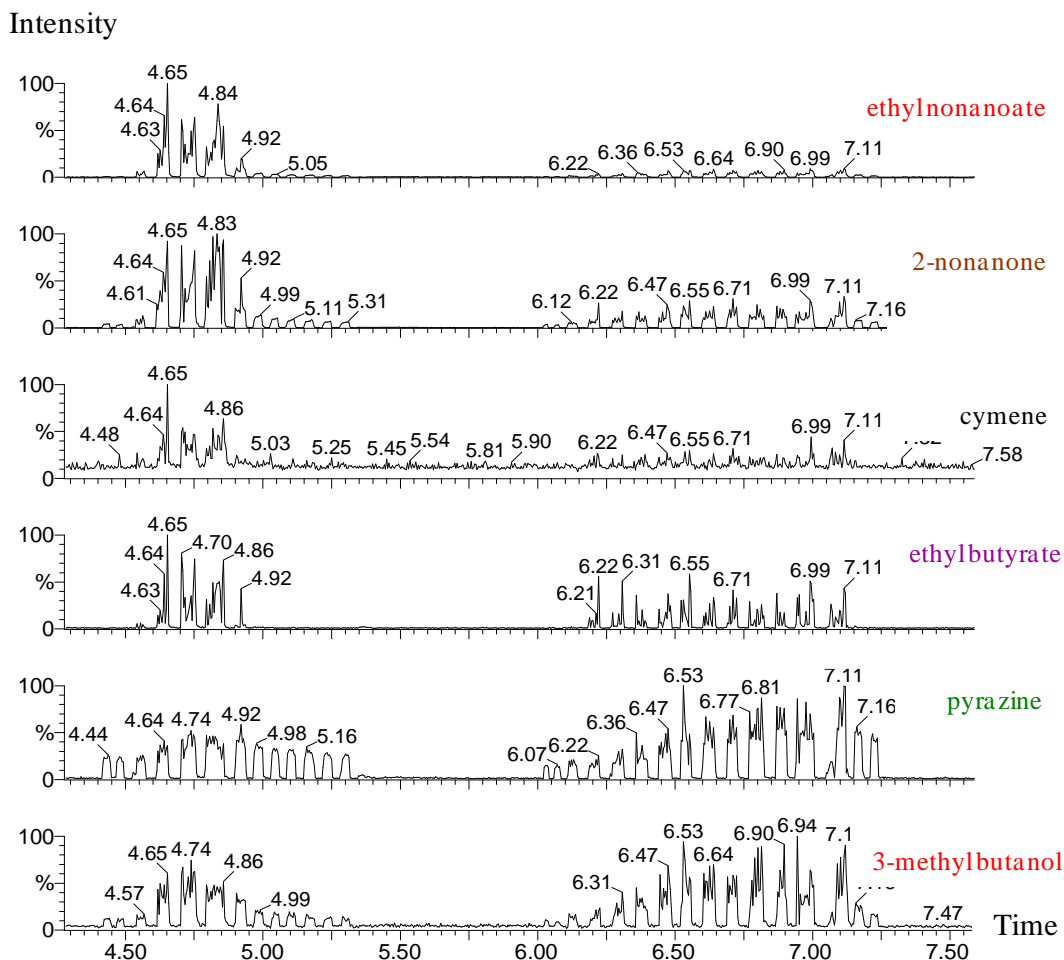


Figure 3.4 APCI-MS trace of aroma release from a single panellist eating a pectin jelly (4 to 5.5. min) and a chewy candy (6 to 7.5 min), both flavoured with the KTP4 flavouring with each compound labelled at the right bottom

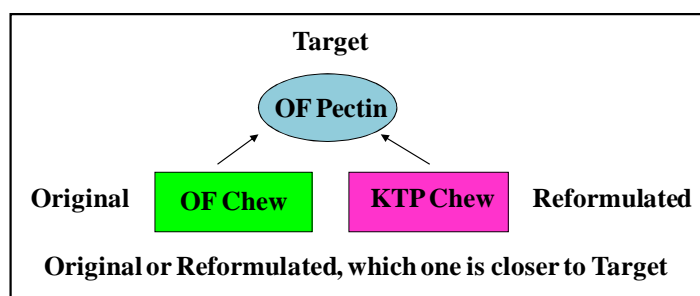
### 3.2.4 Sensory Validation (Reformulated Candies)

The reformulated strawberry flavouring was applied to chewy candy (KTP Chew), and compared with the original flavoured chewy candy (OF Chew) and the original flavoured pectin jelly (OF Pectin). Two types of sensory tests were carried out, one was discrimination test using a duo-trio test and the other was descriptive analysis applying flavour profiles.

#### 3.2.4.1 Duo-trio test for confectionery products

In order to investigate if consumers could differentiate the flavour of the reformulated flavoured chewy candy (KTP Chew) from the original flavoured

chewy candy (OF Chew), a modified duo-trio test (BS-ISO-10399, 2004) was carried out where the original flavoured pectin jelly (OF Pectin) was set as the target flavour (Figure 3.5).



*Figure 3.5 Schematic demonstration for duo-trio test to compare original OF Chew and reformulated KTP Chew with target OF Pectin in terms of flavour.*

The constant-reference technique was applied: OF Pectin as the reference (REF) was presented first and the presentation order of OF Chew (A) and KTP Chew (B) was randomised, such as REF-A-B or REF-B-A. The presentation order among the assessors was designed by Fizz software (Biosystemes, France). Panellists were instructed to taste the product in the order presented: first evaluate the reference sample, then evaluate the two samples that were coded with three randomised digital numbers, finally choose which of the two coded samples matched the reference. They were asked to cleanse their palate between samples using water (Evian natural mineral water, Danone, UK) and crackers (Rakusens's 99% fat free, Rakusen's Limited, UK).

One hundred untrained panellists (staff and students at the University of Nottingham) volunteered to participate in this study. Females comprised 70% of the panellists and the mean age was 28 years. Panellists were forewarned that the candy samples had different textures and were asked to focus on flavour differences. Having eaten the candies they were asked to indicate which one was closer to the flavour of the reference pectin jelly.

#### 3.2.4.2 Descriptive analysis for confectionery products

To compare the perceived differences in strawberry flavour among three products (original pectin jelly, original chewy candy and reformulated chewy candy), five trained flavourists with at least five years working experience first agreed on a set of five attributes to describe the strawberry flavour (sweetness, sourness, fruitiness, juiciness, and greenness). The following form (Figure 3.6) was given to every flavourist to assess each candy product. Panellists rated the intensity of each attribute by marking at the linear scale between 'Low' and 'High'. The marks were converted to numerical values using a scale of 0 to 10 (low to high).

	Low	High
Sweet	-----	
Sour/acidic	-----	
Fruity	-----	
Juicy	-----	
Green	-----	

Figure 3.6 Strawberry flavour profile form for each candy product

Panellists were required to taste the products in the order presented and regularly cleanse their palate between samples with water and crackers. Assessment of the products was undertaken in two replicated sessions and the panellists' scores from these two sessions were averaged.

#### 3.2.4.3 Data analysis for sensory validation of confectionery products

During duo-trio test, all data were recorded and analysed by Fizz software (Biosystemes, France) to determine if a significant result was obtained ( $p < 0.05$ ).

To analyse the results from the sensory descriptive analysis, ANOVA was applied to evaluate if a significant difference existed in any sensory attribute between three types of candy products ( $p < 0.05$ ). The Tukey's test as a tool of multiple comparison test was used to illustrate which product is significantly different.

### **3.3 MODELLING APPROACH**

#### **3.3.1 Yoghurt Products Preparation**

Consumers would prefer low fat flavoured yoghurt product to have a similar flavour to that perceived in the high fat version. Therefore, the flavourings are normally required to be reformulated in low fat yoghurt to target the aroma profile of original high fat yoghurt. It could be difficult to make consistent yoghurt product with lack of knowledge and facilities in the company. Due to these technical limitations of making yoghurt, some commercial plain yoghurt products available in the market were used as the base for added flavourings.

##### *3.3.1.1 Flavouring Addition*

During instrument validation, the KTP4 flavouring was added at 0.2% to both low fat and high fat yoghurt to compare with the low fat yoghurt. The reformulated KTP4 flavouring based on model prediction was added at same level (0.2%) in the low fat yoghurt.

During sensory validation, four commercial flavourings (strawberry, blackcurrant, banana and coffee) were also reformulated using a model. The dosage of banana flavouring and strawberry flavouring with the original and reformulated version was 0.1%; whilst, blackcurrant and coffee flavourings were added at 0.08% for their respective original and reformulated versions.

#### *3.3.1.2 Samples for instrumental validation*

The plain yoghurts from Yeo Valley (North Somerset, UK) with high fat (4.2%) and low fat (0.1%) version were used for instrumental validation. A pot of high fat version (500 g) was used to make triplicate samples with 100 g in each glass bottle (500 ml). The flavouring and the colouring - Allura Red (Blends Ltd, Liverpool, UK) was also added into each bottle and sealed by the screw lid. Similar procedure was applied to a pot of low fat yoghurt products (500 g) to prepare triplicate samples (100 g each) with same amount of flavouring and colouring. The use of colouring was to indicate if the flavouring was well mixed in the yoghurt. The reformulated flavouring was added into another batch of low fat yoghurt following the same process.

A standard mixing procedure was applied to all the samples: rotating up and down using an overhead shaker (Hedoph, Reax 2, power 4) for 30 min and rolling side by side using a Roller Mixer (Thermo Scientific, Tube roller Spiramix 10) for 2 hours. The structure of these yoghurts was disturbed and the texture became thinner after mixing, so they were kept refrigerated (5°C, 12 h) for texture reset prior to measurement.

#### *3.3.1.3 Samples for sensory validation*

The plain yoghurts from Yeo Valley used in previous instrumental analysis tasted different in acidity and texture between its low fat (LF) and high fat (HF) version, which may influence the results of sensory validation. Therefore, another three pairs of plain yoghurts (as shown in Table 3.5) were purchased from local supermarket in order to find the best base for sensory evaluation.

*Table 3.5 Three groups of yoghurts with different fat contents used during sensory product screening session*

<b>Yoghurt Product Name and Type</b>	<b>Fat Content</b>
(I) a) Sainsbury's Greek Style Yoghurt	11%
b) Sainsbury's Greek Style Yoghurt Low Fat	2.7%
(II) a) Onken's Natural Set	3.7%
b) Onken's Natural Fat Free	0.1%
(II) a) Total's Greek Strained Yoghurt	10%
b) Total's 0% Fat Free Greek Strained Yoghurt	0%

During the product screening session, all these plain yoghurt listed in Table 3.5 were sweetened by 10% icing sugar (Silver Spoon, Peterborough, UK) and tasted by five panellists. The first pair (I) was selected by all the panellists to be the best choice as similar base in terms of texture and acidity level. Therefore, flavourings need to be reformulated to compare with its original flavourings in this yoghurt product with 2.7% fat.

The standard yoghurt base (9600 g, 2.7% fat) for duo-trio test was made with 10% icing sugar (960 g). The yoghurt was divided into four amounts of 2500g to add one of the four flavourings at its suggested dosage. The respective colouring was also applied for each flavouring: Allura Red (Blends Ltd, Liverpool, UK) for strawberry flavouring, Lutein (Overseal Foods Ltd, Derbyshire, UK) for banana flavouring, Magento (Overseal) for blackcurrant flavouring, and Caramel (Aromco, UK) for coffee flavouring. All the ingredients were mixed using a Chef Mixer (Kenwood, UK) for 30 min and kept in glass bottles stored in the fridge (5 °C) overnight before the sensory test.

### 3.3.2 Product Variation Analysis

Previously, the variation tests for instrument, product and panellist were carried out for instrument reformulation approach. Since modelling approach avoided

actual measurement by panellists, only the product variation needed to be defined by headspace analysis.

The yoghurt samples made with KTP4 flavouring (Section 3.3.1.2) were used in the headspace analysis with six samples of LF yoghurt and six of HF yoghurt. Every sample (10 g) was diluted with 90 g water and contained in a 300 ml glass bottle and sealed. They were equilibrated at 20 °C for 3 h prior to headspace analysis. The sampling rate from the sample to the APCI source was at 6 mL/min.

### 3.3.3 Modelling Reformulation Method

In addition to the instrumental reformulation approach, a mathematical model developed by Linforth et al. (2010) was also used to guide flavour reformulation. This predictive model was based on total of 345 data points including values from the literature, which studied the effect of fat on in- vivo flavour delivery. Key parameters for model development were the fat contents of the samples themselves and the Log P of the flavour compounds. The lipid effect (LE) value was calculated as the flavour delivery from the fat content (%) of the higher fat content samples (FC2) and the fat content (%) of the lower fat content sample (FC1), as shown in Equation 3.1:

$$\text{Lipid Effect (LE)} = \text{flavour delivery FC2} / \text{flavour delivery FC1} \quad (\text{Equation 3.1})$$

The LE values were modelled using Design Expert (Statease, Minneapolis, US) by Linforth et al. (2010) based on multiple non-linear regression using three values: Log P, FC1 and FC2. The modelling terms were either kept in the model equation if such term has a statistical significance with a probability of  $p < 0.05$ . The final mathematical equation was generated as:



$$LE = 1.41 + 0.082 \times FC1 - 0.037 \times FC2 - 0.11 \times \log P - 0.0069 \times FC1 \times FC2 + 0.052 \times FC1 \times \log P - 0.011 \times FC2 \times \log P + 0.0019 \times FC2^2 - 0.13 \times \log P^2 - 0.00071 \times FC1 \times FC2 \times \log P + 0.00012 \times FC1 \times FC2^2 - 0.0041 \times FC1 \times \log P^2 + 0.0023 \times FC2 \times \log P^2 - 2.7 \times 10^{-5} \times FC2^3 + 0.023 \times \log P^3$$

(Equation 3.2)

The equation had 14 terms in addition to the intercept, including interactive terms between the three factors and quadratic and cubic components. The development process has been described in detail by Linforth et al. (2010).

During flavour reformulation study for yoghurt products, the fat level (FC1 and FC2) in these products was known. Equation 3.2 was used to calculate the LE values for all compounds in the flavouring using their Log P values estimated by EPIsuite<sup>TM</sup>. The original concentration of individual compound in the formula multiplied by its respective LE value would give the adjusted concentration for the reformulated flavouring. This reformulated flavouring would then be applied into the required yoghurt products for instrumental and sensory validation.

### 3.3.4 Instrumental Validation

Yoghurt samples as prepared in Section 3.3.1.2 were used for in-vivo analysis. The settings of APCI-MS measurement were as follows: cone voltage at 18 V and sampling rate at 30 ml/min. Five panellists consumed three replicates of each type of yoghurt product (LF and HF). Moreover, the LF yoghurt with reformulated KTP flavouring was also measured with three replicates by the same five panellists. Panellists consumed the flavoured yoghurt (1 spoon about 5 g) in turn with 15-20 min rest periods and water was provided to cleanse their palate.

To evaluate the in-vivo results measured for LF and HF yoghurt with original KTP4 flavouring, ANOVA was applied to each compound with the significant level of  $p < 0.05$ . To illustrate if there was a significant difference ( $p < 0.05$ ) between the actual measured results with the model predicted values for all six compounds, Paired Samples T-test was applied by SPSS16.00 (SPSS Inc., Chicago, USA). After reformulation, additional ANOVA was used to compare the in-vivo results of reformulated LF yoghurt with the release of original HF yoghurt.

### 3.3.5 Sensory Validation (Reformulated Yoghurts)

Four duo-trio tests were carried out with respect to four flavourings (strawberry, banana, blackcurrant and coffee) with their original version and reformulated version both applied into low fat yoghurt (prepared in Section 3.3.1.3). Each test included three samples: original flavoured yoghurt (REF), same yoghurt as reference (A) and reformulated yoghurt (B). Four duo-trio tests were carried out for four flavourings using similar method described in Section 3.2.4.1. Thirty six panellists (students at the University of Nottingham) attended this study (mean age of 25 years old). The presentation order of four tests involving was balanced by Fizz, and the data were recorded and analysed by Fizz.

## PART II FLAVOUR REFORMULATION

### 4 RESULTS & DISCUSSION

According to the objectives of this study, the results of flavour reformulation through either instrumental approach (Section 4.1) or modelling approach (Section 4.2) will be reported respectively.

#### 4.1 RESULTS OF INSTRUMENTAL APPROACH

##### 4.1.1 Variation Analysis Results

##### 4.1.1.1 Instrument variation

The consistency of the APCI-MS interface in measuring the headspace above aqueous solutions was tested to determine the variation in the basic analytical procedure. Based on four replicates samples and three replicated measurements, the average values of ion intensity based on 12 reps for every compound in KTP4 flavouring were calculated, along with respective standard deviation and percentage coefficient of variation values, as shown in Table 4.1.

*Table 4.1. The average values of ion intensity (AV), the standard deviation (STD) and the percentage coefficient of variation (CV%) calculated from headspace analysis of 12 reps per each compound from the standard solutions of KTP4 flavouring*

Compound	AV (Ion Intensity)	STD	CV%
Pyrazine	82,000	1	2
3-Methyl Butanol	70,000	2	3
Ethyl Butyrate	208,000	2	1
2-Nonanone	1,521,000	32	2
Cymene	39,000	1	3
Ethyl Nonanoate	5,267,000	453	9
Average CV%			3

As shown in Table 4.1, the CV% for every compound between 1% and 9% and the average CV% of all compounds was only approximately 3%. This result indicated that APCI-MS should be a reliable instrument for later flavour reformulation studies.

#### *4.1.1.2 Product variation*

The static headspace concentrations of the dissolved candies were compared within a batch and between batches during static headspace analysis. Based on the average ion intensity values of triplicate samples per batch, the CV% of intra-batch for every compound in KTP4 flavouring could be calculated, as shown in Table 4.2. The average CV% for all compounds per batch was calculated and shown in *italic*, and average CV% for all batches per candy type shown in **bold** indicated the average intra-batch variation for pectin jelly and chewy candy.

Comparing with instrument variation with an average of 3%, product variation within batches of chewy candy was at similar level (4% in average, Table 4.2), but much larger variations exist within batches of pectin jelly (17% in average). Although the same flavouring was added into both candy matrices at same level, the dissolved chewy candy with smaller variation from headspace analysis might due to the lipid in chewy candy to help spread the flavouring within its matrix during manufacturing and lower the HS concentration when chewy candy was dissolved. But this effect was similar for polar and non-polar compounds, so it might be more possible that flavour incorporation within the candy mass in pectin jelly was not as homogeneous as in chewy candy, resulting in larger variations for individual pieces within one batch.

Table 4.2 The coefficient of variation (CV%) of intra-batch variation based on triplicate samples per batch calculated for six compounds in the KTP4 flavouring of every batch of pectin jelly (Batch A, B, C, D, E) and chewy candy (Batch A, B, C) during static headspace analysis. The average CV% of all compounds per batch and average CV% for all batches per candy type were shown in italic and broad respectively.

<u>Within Batches</u> (CV%)	<b>Pectin Jelly</b>					<b>Chewy Candy</b>		
Compounds	Batch A	Batch B	Batch C	Batch D	Batch E	Batch A	Batch B	Batch C
Pyrazine	8.0	28.0	15.5	16.1	10.1	1.6	3.3	0.9
3-Methyl Butanol	9.3	14.5	13.3	15.9	11.2	1.4	6.7	2.8
Ethyl Butyrate	33.0	36.3	28.4	29.3	23.5	2.2	6.7	3.0
2-Nonanone	10.8	28.7	17.8	12.6	13.5	2.8	6.1	2.1
Cymene	9.0	31.4	9.9	11.9	5.7	3.9	4.6	5.1
Ethyl Nonanoate	18.1	15.2	17.6	7.1	9.9	2.3	8.5	4.8
<i>AV (CV%) per batch</i>	<i>15</i>	<i>26</i>	<i>17</i>	<i>15</i>	<i>12</i>	<i>2</i>	<i>6</i>	<i>3</i>
<b>AV (CV%) per candy type</b>	<b>17</b>					<b>4</b>		

Besides intra-batch variations shown in the table above, the results of inter-batch variations for pectin jelly and chewy candy were analysed and the data is summarised in Table 4.3.

Table 4.3. The mean values (AV) were the average ion current values divided by 1000 for five batches pectin jelly and three batches chewy candy. The standard deviation (STD) and the coefficient of variation (CV%) were calculated to show inter-batch variations of candy solutions in static headspace analysis.

<u>Between Batches</u>	<b>Pectin Jelly</b>			<b>Chewy Candy</b>		
Compounds	AV	STD	CV%	AV	STD	CV%
Pyrazine	9	3	31	68	10	14
3-Methyl Butanol	11	3	27	68	4	6
Ethyl Butyrate	12	5	47	115	46	40
2-Nonanone	113	35	31	45	10	23
Cymene	14	7	49	1732	240	14
Ethyl Nonanoate	1303	388	30	1386	150	11
<b>AV (CV%)</b>	<b>36</b>			<b>18</b>		

Generally, there was a significant difference of CV% between pectin jelly and chewy candy ( $p < 0.05$ ) based on ANOVA. According to Table 4.3, the average batch-to-batch variation for pectin jelly (36%) was double the variation of chewy candy (18%). The difference in preparation methods of these two candies could be the main explanation. Both methods involved adding the flavouring to the semi-finished candy as a propylene glycol solution and further mixing of the candy mass. Since the candy mass at this stage is highly viscous and hot, obtaining efficient mixing between the small volume of added flavouring and the larger bulk of the candy mass is difficult. For chewy candy, the flavouring was mixed into the candy mass by vigorous mixing using a metal beater for 2 min and then rolling by hand continuously for 5 min. Whilst, flavouring for pectin jelly was added after the hot candy mass was prepared through quickly stirring with a wooden spoon by hand. There is a time limit for pectin jelly because the candy mass cools during the mixing stage and soon becomes very viscous, which prevents effective mixing. As a result, considerably larger variations were involved in the different production batches of pectin jelly than batch-to-batch variations of chewy candy.

Comparing with instrument variation at 3% in average, the product variation (within and between batches) was at much higher level for both pectin jelly (17% and 36%) and chewy candy (4% and 18%). Due to the nature of candy manufacturing process, the considerable inter-batch product variation is inevitable, even if the critical control point for mixing flavourings with candy mass has been managed as thoroughly as possible. During the reformulation study, several batches of test samples were combined to minimise the impact of inter-batch variations.

#### 4.1.1.3 Panellist variation

The variation between panellists from APCI-MS in-vivo analysis is the major challenge for flavour reformulation using instrumental approaches. Variation in the mastication patterns, breathing and swallowing rates of human panellists is well established (Blissett et al., 2006) and is known to affect flavour release. Gierczynski et al. (2011) reviewed the inter-individual variability of subjects, mainly chewing behaviour, saliva rate and composition and their influence on aroma release.

An example of ethyl butyrate release for six panellists during pectin jelly and chewy candy consumption is illustrated in Figure 4.1.

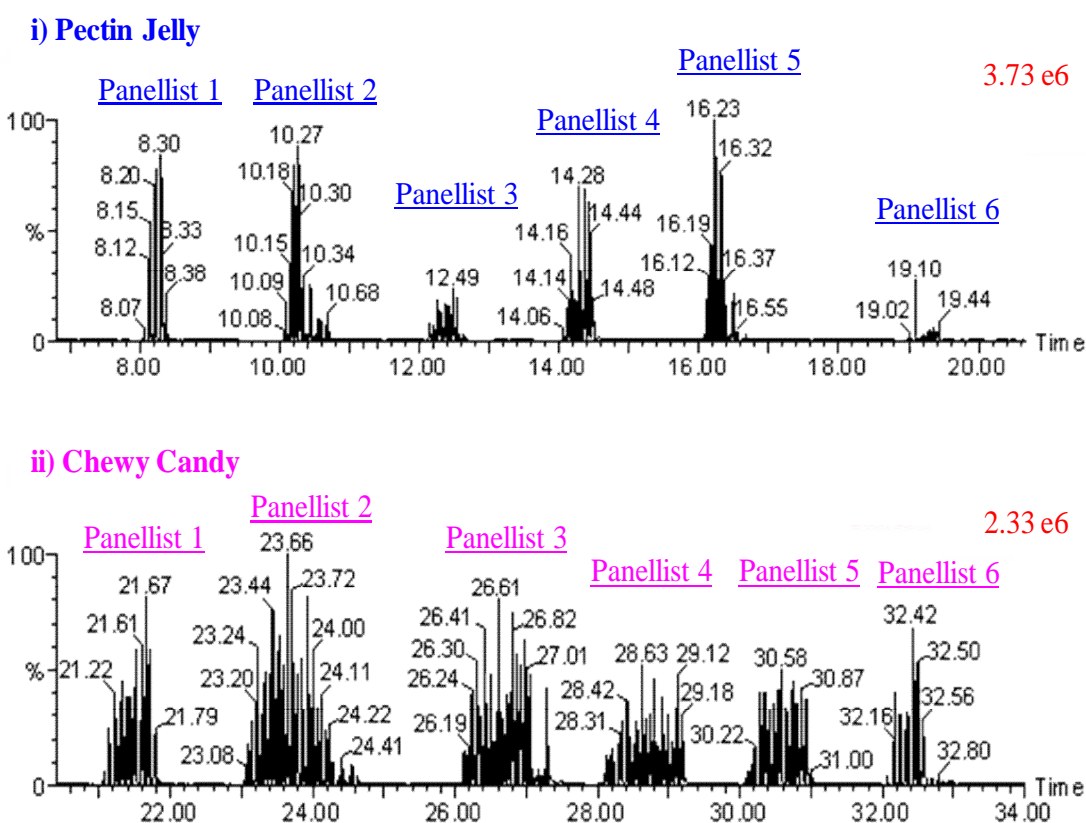


Figure 4.1 Release of ethyl butyrate for six panellists (1-6) during consumption of i) pectin jelly starting at time 8, 10, 12, 14, 16 and 19 min, and ii) chewy candy starting at time 21, 23, 26, 28, 30 and 32 min. The maximum ion intensity is 3.73 e6 and 2.33 e6 for pectin jelly and chewy candy respectively.

Comparing the overall release pattern between pectin jelly and chewy candy across all panellists shown in Figure 4.1, the in-vivo release mainly differs in the following aspects: i) the maximum intensity release for pectin jelly (3.76 e6) was higher than chewy candy (2.33 e6); ii) pectin jelly generally had shorter release duration (< 1 min) than chewy candy (>1 min); iii) time to the maximum intensity was normally quicker for pectin (~20 s) compared to chewy candy (~60 s).

Different oral processing resulting from distinct texture and fat content differences between these two candies could be the main factor affecting release. Potential factors are: i) ethyl butyrate could be disposed with fat phase in the chewy candy resulting in the reduced release intensity comparing with pectin jelly; ii) soft pectin jelly could be broken down into smaller pieces easily during eating to allow rapid release of ethyl butyrate from the matrix and then swallowed immediately resulting in a shorter release duration, while hard chewy candy was sticky and adhered to the teeth which took much more time to chew, so its release curve is more consistent and over a longer time; iii) quicker oral processing for pectin jelly provided quicker release of aroma compounds, so the time to achieve its maximum intensity is shorter than the time taken for the longer processing to release aromas from chewy candy.

Regarding to panellist-to-panellist variations, panellists 3 and 6 in Figure 4.1 illustrated considerable lower release in pectin jelly than the other four panellists. The release intensity during consumption of chewy candy across six panellists was more reproducible than pectin jelly. This is because soft pectin jelly with rapid aroma release profile might be more dependent on how people chew it. The



chewy candy might generate a more consistent chewing pattern as the panellists consume it.

Comparing with inter-individual differences, intra-individual differences are much smaller. Figure 4.2 shows the release of the six compounds in the KTP4 flavouring when a single panellist consumed three replicates of the pectin jelly.

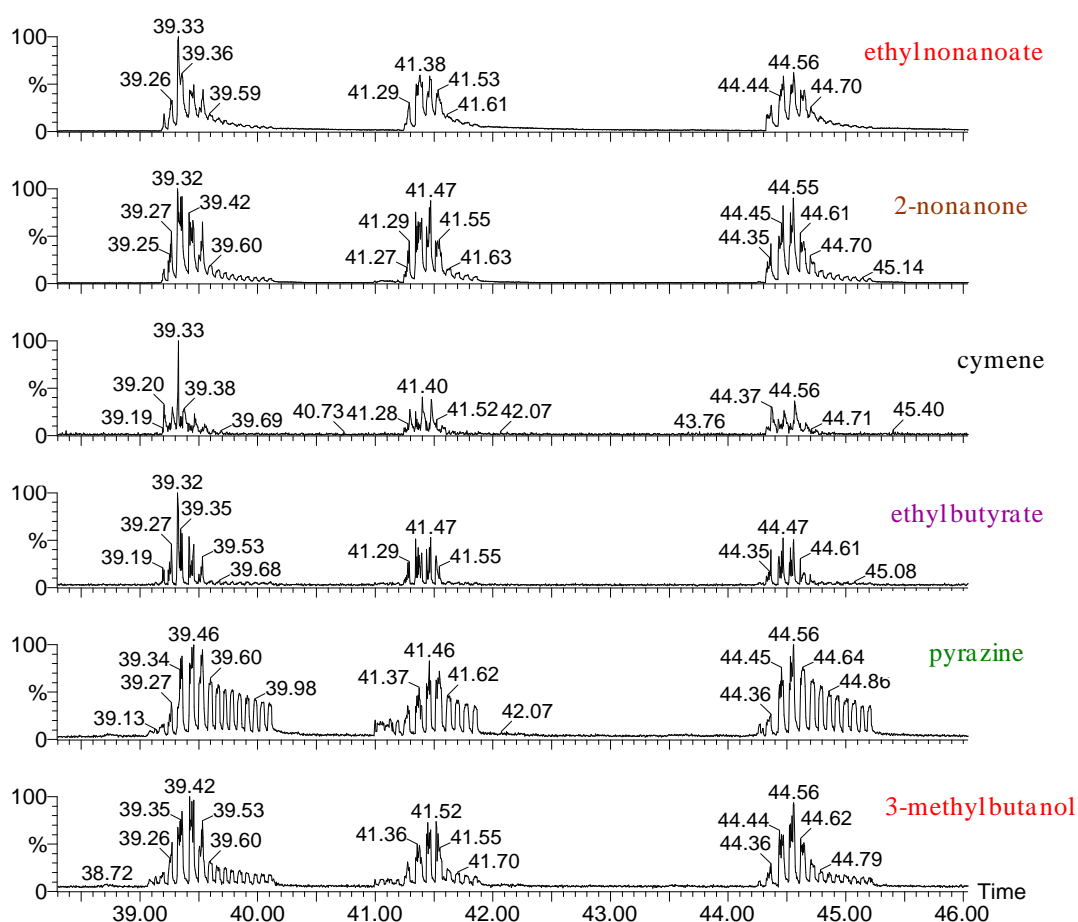


Figure 4.2 Release of six compounds in the KTP4 flavouring for a single panellist eating three replicates of pectin jelly (39-40 min, 41-42 min, and 44.2-45.2 min)

As shown in Figure 4.2, both the shape and extent of release were comparable across the three replicates of pectin jelly for all the compounds. Therefore, it is essential to assess the level of variations for every panellist during eating these two types of candies. Based on the I<sub>max</sub> results for every compound in KTP4 flavouring for each types of candy, the variation of every panellist for all six

compounds was calculated with the expression of CV% for respective pectin and chewy candies (Table 4.4).

*Table 4.4 Variation of aroma release (Imax results) from both pectin and chewy candies with KTP4 flavouring for i) every 10 panellist consumption of three reps of pectin and chewy candies (10 x 3 system) and ii) every 4 panellist consumption of nine reps of each candy (4 x 9 system). The mean CV% for each system and the overall CV% for two systems were calculated.*

Panellist	Sample Replicates	Pectin %CV	Chewy %CV
1	3	22	14
2	3	38	60
3	3	11	23
4	3	12	35
5	3	30	7
6	3	46	23
7	3	38	12
8	3	46	13
9	3	6	3
10	3	60	23
Mean CV% (10 x 3 system)		<b>31</b>	<b>21</b>
1	9	24	20
2	9	43	19
3	9	28	12
4	9	25	15
Mean CV% (4 x 9 system)		<b>30</b>	<b>17</b>
<b>Overall Mean CV% (all panellists)</b>		<b>31</b>	<b>20</b>

As shown in Table 4.4, one panellist (panellist 9) illustrated very consistent release from the pectin and chewy candies with CV% values of 6% and 3% respectively. These values were actually lower than the candy-to-candy flavour content variation measured previously (17% and 4% within batch variation of pectin and chewy candy). Other panellists showed values from 7% to 60%. These variations are presumably due to the different ways that individual panellists masticate the candies. With the pectin jelly, the degree of fragmentation is important in creating new surfaces for aroma release and therefore mastication rate is a potential factor, along with saliva flow rates and swallowing behaviour.

These factors were not measured in this experiment as the goal was to measure overall variation and release differences rather than the cause of variation.

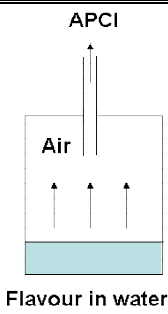
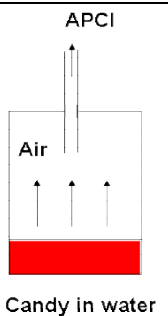
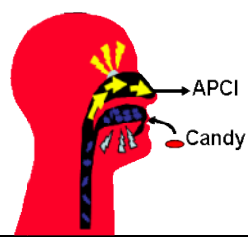
Table 4.4 also shows average variation in the 10 x 3 and the 4 x 9 system for pectin jelly was 31 and 30 %, and for chewy candy was 21 and 17 %. The t-test for ten paired results in the 10 x 3 system indicated that there is no significant difference ( $p < 0.05$ ) observed on flavour release variation between these two candies for different panellists. Whilst in the 4 x 9 system, the panellist variation in flavour release between two products was significantly different ( $p < 0.05$ , paired t-test).

The choice of using more panellists or increasing the number of replicated samples is dependent on the purpose of the study. If reformulation of flavouring is the aim, the 10 x 3 system might be preferred to cover more panellists with less sample replicates; while if the aim of the study is on the texture effect on aroma release with different products, more sample reps in the 4 x 9 system should be a better choice to distinguish different texture properties with minimised panellist variations in aroma release.

## **Summary**

The results of the variation data for reformulation through instrumental (APCI-MS) approach is summarised in Figure 4.3. Taking the product variation within or between batches into account, the total variations with the sum of all three sources for pectin and chewy candy were calculated as 51-70% and 27-41% respectively. This was a concern on whether if flavourings could be reliably reformulated with this range of variation or if there was another approach to provide more consistent data for in-vivo release analysis. Therefore, further

analysis was carried out and a standard protocol was developed to verify the minimum number of panellists and samples required, as discussed in the following section.

<b><u>Sources of Variation</u></b>	<b><u>Demonstrations</u></b>	<b><u>Results</u></b>
<b>i) <u>Instrument</u></b>		- Results of instrument variation:  $CV\% = 3\%$
<b>ii) <u>Product</u></b>		- Results of product variation:  $CV\%$ within batches of pectin = 17%; $CV\%$ within batches of chewy = 4%. $CV\%$ between batches of pectin = 36%; $CV\%$ between batches of chewy = 18%.
<b>iii) <u>Panellist</u></b>		- Results of panellist variation:  $CV\%$ during pectin consumption = 31% $CV\%$ during chewy consumption = 20%

*Figure 4.3 Summarised results of variation analysis including i) instrument variation, ii) product variation, and iii) panellist variation*

#### 4.1.2 Standard Protocol Development

A consistent sample preparation could reduce some variation of measurement, but some variations are inherent, like from different chewing styles between individuals. To reduce this panellist variation, Shojaei et al. (2006a) used large number of panellists up to 90 to consume each sample in quadruplicate, but this makes the testing procedure laborious, slow and expensive. So this study attempted to obtain aroma release data representative of a larger group with a

minimum number of panellists and sample replicates. Therefore, further analysis was carried out to find out the minimum number of panellists and replicates required based on the previous two test systems: 10 panellists ate 3 replicates of each candy (10 x 3 system) and 4 panellists ate 9 replicates per sample (4 x 9 system).

#### 4.1.2.1 Number of panellists required

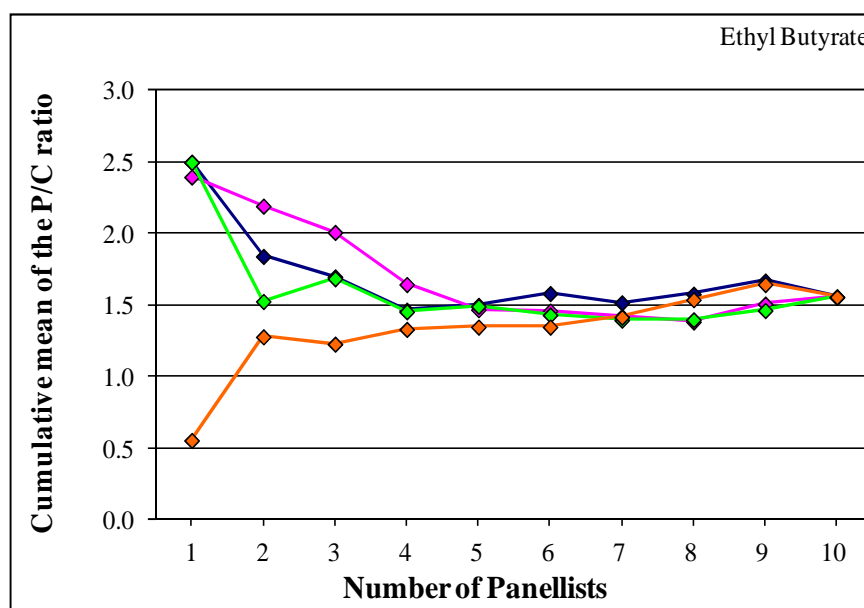
From the release curves, the maximum ion intensity (Imax) for every panellist and each replicate was recorded. In the 10 x 3 system, the average Imax for one panellist consuming three replicates of one candy were calculated. The ratio between the average Imax of pectin and chewy candies was calculated for every panellist as:  $\text{Imax (Pectin jelly)} / \text{Imax (Chewy candy)}$ , which is called “P/C ratio”. Taking ethyl butyrate as example, the P/C ratios calculated for 10 panellists are shown in the first two columns in Table 4.5.

*Table 4.5 The P/C ratios for ethyl butyrate of individual panellist from A to N, and the cumulative mean P/C values as panellists are added together in a random order.*

Panellist	P/C ratio	Cumulative Panellist	Cumulative Mean P/C
A	2.50	A	2.50
K	1.18	AK	1.84
M	1.41	AKM	1.70
P	0.77	AKMP	1.47
R	1.64	AKMP R	1.50
S	1.99	AKMP RS	1.58
J	1.13	AKMP RSJ	1.52
O	2.00	AKMP RSJO	1.58
X	2.40	AKMP RSJOX	1.67
N	0.56	AKMP RSJOXN	1.56

To answer the question of how many panellists are needed to obtain a result that is representative of the larger group, the P/C ratios of the panellists were added together and mean values taken as the value for each panellist was added. This is known as “cumulative mean”, as shown in the last column in Table 4.5. The order of panellists was picked at random, as one example of a “cumulative mean” calculation of the P/C ratio for ethyl butyrate starting with one panellist and finishing with ten panellists.

Moreover, different orders of panellists could be chosen at random. Figure 4.4 illustrated this process where cumulative mean calculations were carried out using four random orders of panellists for the release ratio of ethyl butyrate.



*Figure 4.4 Cumulative mean of the P/C ratio of ethyl butyrate using total of ten panellists. Each line represents a cumulative mean calculation for four random orders of panellists*

It is clear from Figure 4.4 that the P/C ratios start to converge after the values from four panellists have been averaged and this result was found irrespective of the order of calculation.

The P/C ratios for the other compounds, like ethyl hexanoate (Figure 4.5) showed the same behaviour. Therefore, five panellists were proposed to be the minimum number required for reformulation studies by APCI-MS.

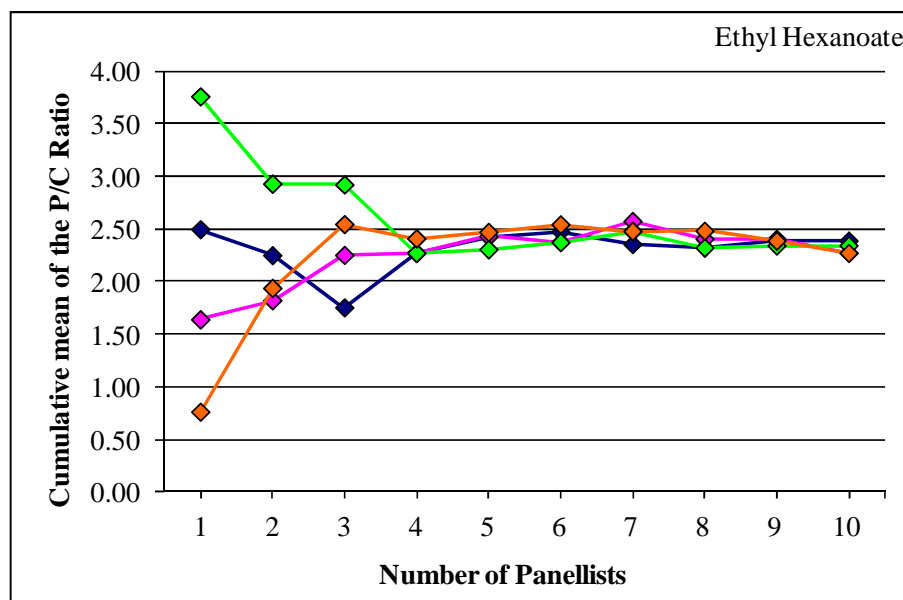


Figure 4.5 Cumulative mean of the P/C ratio of ethyl hexanoate using total of ten panellists. Each line represents a cumulative mean calculation for four random orders of panellists

Further study (Linforth et al., 2011) using 50 panellists indicated strong correlations in the intensity of volatile release across sample types. Panellists who released high concentrations of volatiles into their breath from one sample type would typically do so for another. Equally, panellists who released lower amounts from one sample type would also release less from others. The use of five panellists with their Pectin/Chewy release ratios should provide sufficient data for flavour reformulation. In addition, the use of the release ratio minimises the release differences between panellists.

#### 4.1.2.2 Number of samples required

Similar methods to calculate the cumulative mean (see Table 4.5) could be carried out to find out the number of samples required in the 4 x 9 system, where 4

panellists ate 9 replicates per sample. Two examples are illustrated by the cumulative mean of the P/C ratio for 3-methyl butanol of Panellist L (Figure 4.6) and for 2-nonanone of Panellist N (Figure 4.7).

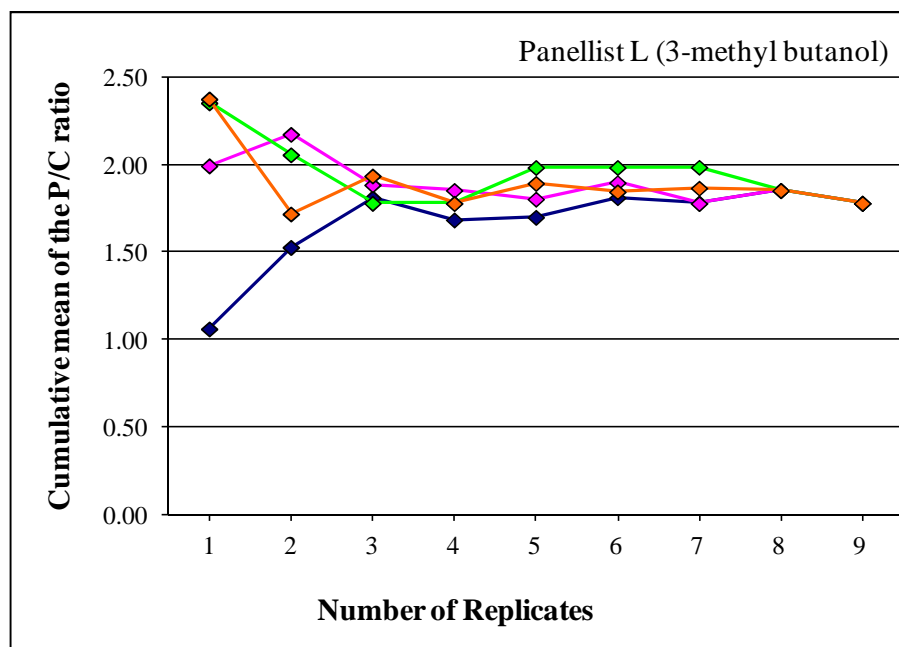


Figure 4.6 Cumulative mean of the P/C ratio for 3-methyl butanol of Panellist L eating nine replicate samples for pectin and chewy candies. Each line represents a cumulative mean calculation for four random orders of replicates.

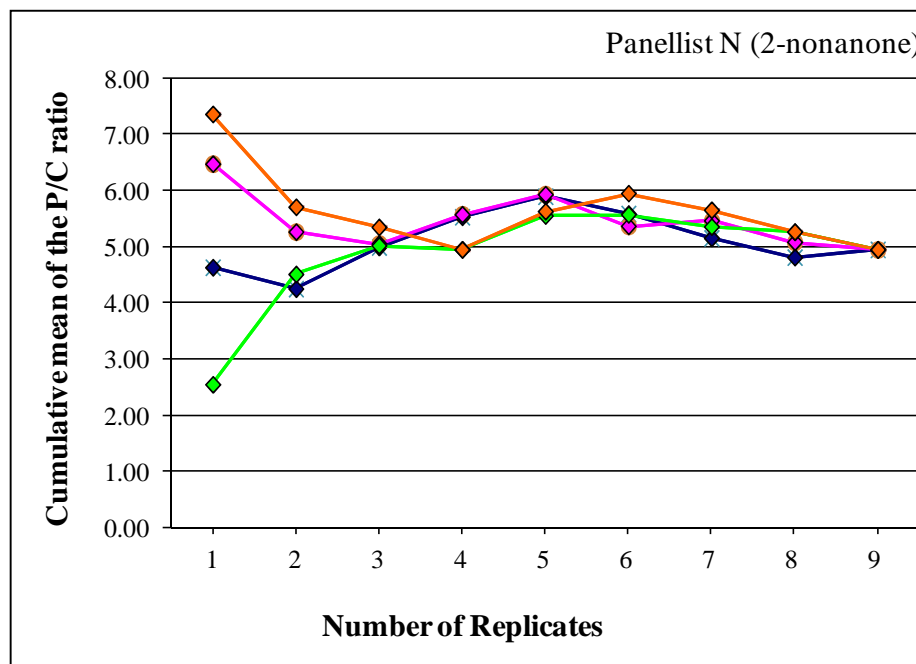


Figure 4.7 Cumulative mean of the P/C ratio for 2-nonanone of Panellist N eating nine replicate samples for pectin and chewy candies. Each line represents a cumulative mean calculation for four random orders of replicates.



Regarding to Figure 4.6 and Figure 4.7, the minimum number of replicated samples required should be three because this is the point of convergence. Other compounds in the KTP4 flavouring illustrated similar patterns. Consequently, a standard protocol for flavour reformulation studies through instrument approach was developed, that is using five panellists consuming three replicates of each product.

#### 4.1.3 Reformulation Development for Instrumental Approach

A strawberry flavouring consisting of nine compounds was added to pectin jelly and chewy candy. The aim of this study was to reformulate the strawberry flavouring for chewy candy with similar release to pectin jelly through instrumental measurement.

##### *4.1.3.1 Results of headspace analysis of individual flavour compounds*

In order to reformulate this strawberry flavouring, it was necessary to check if all nine compounds could be detected by APCI-MS. The standard solutions of individual compounds were made previously for the static headspace analysis. The results indicated that eight out of nine compounds were detected with their representative peaks shown in the chromatogram. The exception was furaneol, in which HS chromatogram showed lots of large noisy peaks but the expected peak at  $m/z$  129 cannot be detected. Moreover, it was also found that the APCI ion for butyric acid ( $m/z$  89) was overlapped by a fragmentation ion of ethyl butyrate, so the peak at 89 could not represent butyric acid when the strawberry flavouring was analysed.

Therefore, seven out of nine compounds from the strawberry flavouring could be detectable during APCI-MS headspace analysis. However, it is important to investigate if these compounds are at detectable level during in-vivo analysis.

#### *4.1.3.2 Results of in-vivo analysis during candy consumption*

Using in-vivo analysis, only four compounds were detected during consumption of strawberry flavoured candies (i.e., ethyl butyrate, ethyl hexanoate, cis-3-hexanol and methyl cinnamate). The concentration of the other three compounds in the candy (diacetyl, gamma-decalactone and hedione) was found to be at too low a level to be detected by APCI-MS.

In order to predict the behaviour of these undetectable compounds, some alternative detectable compounds were chosen based on similar hydrophobicity. This is based on the assumption that the impact of fat on aroma release is dependent on the hydrophobicity of the compound, i.e., compounds at similar hydrophobicity (Log P) should have a similar fat effect on aroma release.

According to Table 4.6, the first three undetectable compounds were diacetyl, furaneol and butyric acid with Log P values of -1.34, 0.82 and 1.07. These were replaced by pyrazine, methyl acetate and ethyl acetate with Log P values of -0.06, 0.37 and 0.86. Moreover, gamma-decalactone in the alternative flavouring was added at higher concentrations that could be detected. Since the Log P of ethyl hexanoate (2.83) is close to the Log P of hedione (2.98), release result of ethyl hexanoate could also indicate the behaviour of hedione.

Table 4.6 Alternative compounds were selected based on similar Log P values of undetectable compounds for in-vivo analysis

Undetectable Compounds	Log P	Alternative Compounds	Log P
diacetyl	-1.34	pyrazine	-0.06
furaneol	0.82	methyl acetate	0.37
butyric acid	1.07	ethyl acetate	0.86
gamma-decalactone	2.57	gamma-decalactone	2.57
hedione	2.98	ethyl hexanoate	2.83

These alternative compounds were mixed with propylene glycol and added into pectin jelly and chewy candy. The in-vivo release of these five compounds from these two types of candy was measured. The calculated release ratios between pectin and chewy candy for these five compounds represented the P/C ratios for their respective undetectable compounds at similar Log P values, as shown in Table 4.7. For example, the calculated P/C ratio of 0.5 for pyrazine was the predicted P/C ratio for diacetyl. Similarly, the predicted P/C ratios for other four compounds (shown in blue, Table 4.7) were estimated from their respective alternatives (shown in red). In addition, the P/C ratios of four detectable compounds (shown in black) in strawberry flavouring were calculated.

Table 4.7 The P/C ratios ( $I_{\max}$  of pectin/  $I_{\max}$  of chewy candy) of alternative compounds (shown in red) to predict the ratio for undetectable compounds (shown in blue) at similar Log P values, plus the P/C ratio of detectable compounds in strawberry flavouring (shown in black)

Alternative Compounds	Log P	P/C ratio	Strawberry Flavouring	Log P	P/C ratio
pyrazine	-0.06	0.5	diacetyl	-1.34	0.5
methyl acetate	0.37	1.2	furaneol	0.82	1.2
ethyl acetate	0.86	1.1	butyric acid	1.07	1.1
			cis-3-hexenol	1.61	1.0
			ethyl butyrate	1.85	1.6
			methyl cinnamate	2.36	1.8
$\gamma$ -decalactone	2.57	2.0	$\gamma$ -decalactone	2.57	2.0
			ethyl hexanoate	2.83	2.4
ethyl hexanoate	2.83	2.4	hedione	2.98	2.4

Regarding to all nine compounds, the data in Table 4.7 illustrated that the P/C ratio generally increases with the increase of Log P value. The most hydrophilic compound (pyrazine), which might be more repulsed by lipid, released double the amount in the fat-containing chewy matrix than non-fat pectin matrix ( $P/C = 0.5$ ), so only half the amount was required in chewy candy to achieve the same release as pectin jelly. Whilst, the most hydrophobic compound (ethyl hexanoate), which might be bound, adsorbed or solubilised by lipid, released 2.4 times more in pectin jelly than chewy candy ( $P/C = 2.4$ ), so 2.4 times of its original concentration should be added in chewy candy to reformulate its release from pectin jelly. Similarly, reformulated strawberry flavouring for chewy candy could be prepared involving every compound at its respective concentration calculated from its original level multiplied by its respective P/C ratio. Since the concentration of each compound in the strawberry flavouring is confidential, the accurate amount of reformulated level will not be disclosed.

#### 4.1.4 Sensory Validation (Reformulated Candies)

##### *4.1.4.1 Results of duo-trio test for confectionery products*

According to tables in BS-ISO-10399 (2004), with 72 out of 100 panellists having chosen the reformulated chewy candy, there was a significant difference ( $p < 0.001$ ) between the reformulated and original chewy candy flavour, and hence the reformulated chew was closer to that of the reference pectin jelly in terms of strawberry flavour. Therefore, this sensory test validated that the perception difference is directly linked to aroma release difference measured by APCI-MS when the in-mouth release is similar between products.

#### 4.1.4.2 Results of descriptive test for confectionery products

The profile for the three types of candies is summarised in Figure 4.8. The profile for original flavoured chewy candy (OF Chew) was the reduced for all attributes. The overall profile of the reformulated chewy candy (KTP Chew) was closer to the profile of OF Jelly than OF Chew, thus explaining why the reformulated chewy candy tasted closer to the target OF pectin by most un-trained panellists in the previous duo-trio test. ANOVA results indicated that the reformulated chewy candy was not significantly different to the pectin jelly in terms of sourness, fruitiness and juiciness of flavour ( $p > 0.05$ ). The attributes of sourness, fruitiness and juiciness were considered to be the standard in terms of perceived strawberry flavour (Yang et al., 2011).

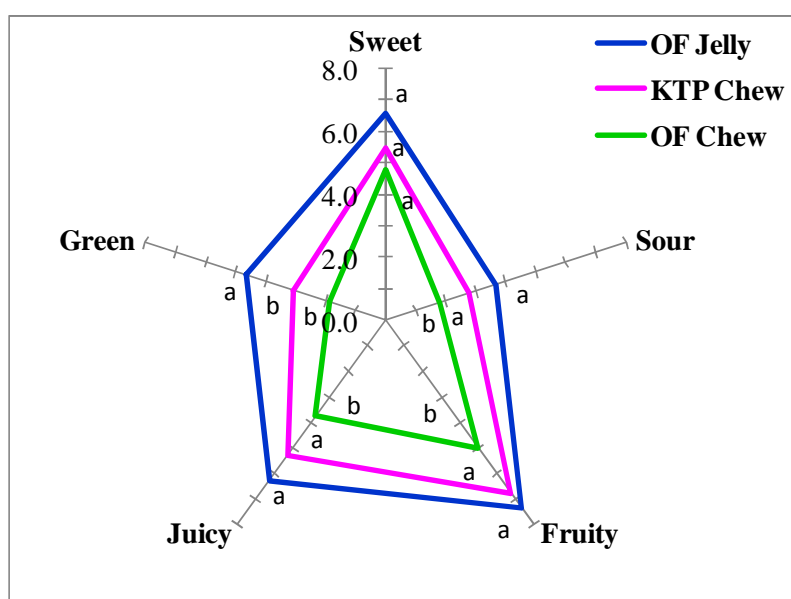


Figure 4.8 Spider diagram showing the intensity of perceived strawberry flavour attributes in the three candies (OF Jelly, KTP Chew and OF chew). Data are the mean of five panellists obtained with duplicate samples in two sessions and different letters (a, b) indicated a significant different at  $p < 0.05$ .

There was no significant difference in terms of sweetness between all three candies ( $p > 0.05$ ). In terms of “greenness”, KTP Chew and OF Chew were

perceived as significantly different from OF Jelly ( $p < 0.05$ ). So some further improvements in reformulating this flavour could be made here.

Overall, reformulation of the strawberry flavouring based on the I<sub>max</sub> ratio of every compound released from pectin jelly and chewy candy (i.e., P/C ratio) was successful. This approach was proved to alter the sensory profile of the chewy candy and bring it closer to the standard profile of the pectin jelly, which was considered to deliver a high quality strawberry flavour.

However, for more complicated flavourings, like a typical blackcurrant flavouring consisting of more than twenty compounds, only five of them were detected during in-vivo analysis by APCI-MS (not shown). This was an example to illustrate the challenge faced by direct measurement of more complex flavourings, so the model prediction without actual measurement may offer an easier approach to reformulate these types of flavourings.

## 4.2 RESULTS OF MODELLING APPROACH

### 4.2.1 Results of Product Variation

First of all, the product variation was checked by headspace analysis of aroma solutions of yoghurt products. The aroma release results were collected for six replicated samples, and the percentage of coefficient of variation (CV%) was calculated from the maximum ion intensity measured for each sample (shown in Table 4.8).

*Table 4.8 Product variation (CV%) using static headspace analysis for low fat (LF) and high fat (HF) yoghurt with KTP4 flavouring*

<b>Compounds</b>	<b>LF yoghurt (CV%)</b>	<b>HF yoghurt (CV%)</b>
pyrazine	3%	3%
3-methyl butanol	6%	1%
ethyl butyrate	3%	4%
2-nonanone	4%	3%
para-cymene	4%	3%
ethyl nonanoate	6%	6%
<b>AV (CV%)</b>	<b>4%</b>	<b>3%</b>

The average product variation for KTP4 flavouring was 4% and 3% for low fat and high fat yoghurt respectively. This level of variation indicated that KTP4 flavouring was well mixed in the all yoghurt samples, which could be used for modelling reformulation studies.

### 4.2.2 Reformulation Development for Modelling Approach

The reformulation approach based on the mathematic model was demonstrated on yoghurt products at 0.1% fat (FC1) and 4.2% fat (FC2) both flavoured with KTP4 flavouring. The Log P of every compound in the KTP4 flavouring could be found

in Table 3.1. The lipid effect (LE) values for all six compounds were calculated from the Equation 3.2 (Section 3.3.3), as shown in Table 4.9.

*Table 4.9 The results of lipid effect (LE) calculated from the model equation for every compound in KTP4 flavouring when FC1 = 0.1% and FC2 = 4.2%.*

<b>Log P</b>	<b>Compound</b>	<b>Lipid Effect</b>
-0.06	pyrazine	1.30
1.26	3-methyl butanol	0.95
1.85	ethyl butyrate	0.73
2.71	2-nonanone	0.44
4.00	cymene	0.21
4.30	ethyl nonanoate	0.22

Generally, the above results illustrated that more hydrophobic aroma compounds (Log P= 2.71, 4.00 and 4.30) would be influenced by the fat content in the yoghurt to a larger extent than hydrophilic compounds (Log P = -0.06 and 1.26). These more hydrophobic compounds (2-nonanone, cymene, and ethyl nonanoate) were reformulated at a much lower level (44%, 21% and 22% of its original level respectively) in LF yoghurt to achieve the similar release from HF yoghurt.

These predicted LE values could be compared with actual measured results from in-vivo release ratios between these two yoghurt products as shown in the following section.

#### 4.2.3 Instrumental Validation

Using the average I<sub>max</sub> results from five panellists with three replicated samples, the in-vivo release results for every compound from low fat yoghurt (LF) were normalised to those in high fat yoghurt (HF) as 100%, as shown in Figure 4.9.



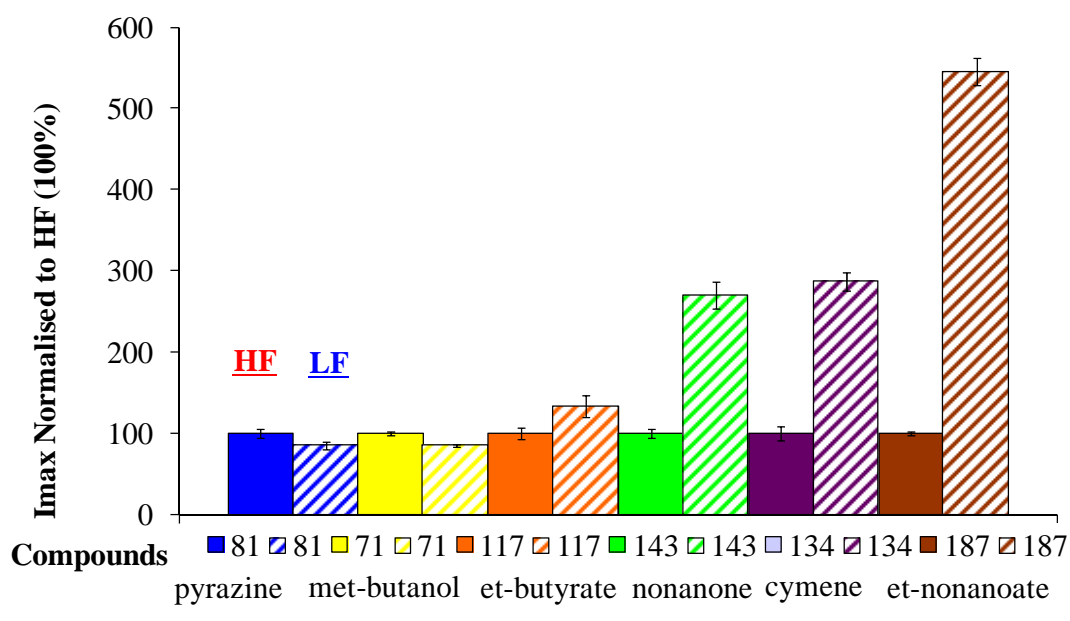
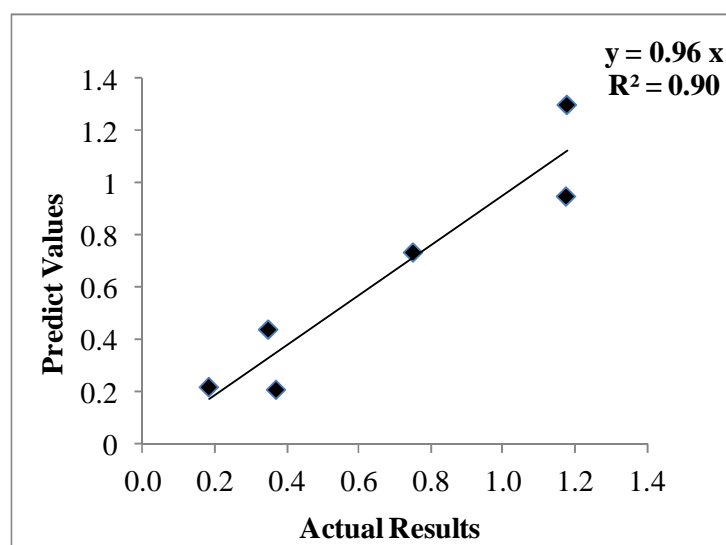


Figure 4.9 In-vivo release of KTP4 flavouring showing the  $I_{max}$  of high fat yoghurt (HF) as 100% (with filled bar) and normalised  $I_{max}$  for low fat yoghurt (LF) (with striped bar). Different colour represents different compounds, labelled by the APCI-MS ion mass and its name underneath. Error bars represented the standard error calculated for each compound.

The compounds in Figure 4.9 from left to right are arranged in the order of their Log P values from low to high. ANOVA showed the release difference between LF and HF was significantly different for 2-nonanone ( $p < 0.01$ ), cymene ( $p < 0.01$ ) and ethyl nonanoate ( $p < 0.001$ ). Similar to the predicted lipid effect values shown in Table 4.9, there was a clear trend of increasing release in low fat yoghurt with increasing Log P. More hydrophobic compounds from 2-nonanone to ethyl nonanoate (Log P from 2.71 to 4.30) showed more than twice the release in LF compared to HF yoghurt. This finding was consistent to other studies (van Ruth et al., 2000; Carey et al., 2002), which proved that the release of hydrophobic compounds are more affected by the level of fat in food products.

Based on the  $I_{max}$  differences shown in Figure 4.9, the release ratio between HF and LF yoghurt was calculated for every compound in KTP4 flavouring. These

ratios from actual measurement were plotted against the predicted LE values calculated from model equation, as shown in Figure 4.10.



*Figure 4.10 Correlation between actual results for the release ratio between HF and LF yoghurt measured from in-vivo analysis and predicted LE values calculated from the model equation for six compounds in the KTP4 flavouring*

The squared correlation coefficient of 0.90 indicates a good model fit ( $r = 0.95$ ), and the slope of 0.96 (close to one) indicated that the model could give close predictions with the actual measured results by in-vivo analysis. Additional data analysis by Paired Sample T- test indicated no significant difference between actual results and predicted values ( $p > 0.05$ ). Hence, this model was shown to be a useful tool to predict the lipid effect on aroma release from yoghurt with different fat content.

Consequently, the model removes the need for actual instrumental measurement to reformulate flavourings. The KTP4 flavouring that was reformulated using the predicted values was applied into LF yoghurt. The in-vivo release from the reformulated LF yoghurt was measured and normalised to the release from the original HF yoghurt (as 100%). The normalised I<sub>max</sub> data for reformulated LF

yoghurt compared to the I<sub>max</sub> data for original HF yoghurt are shown in Figure 4.11.

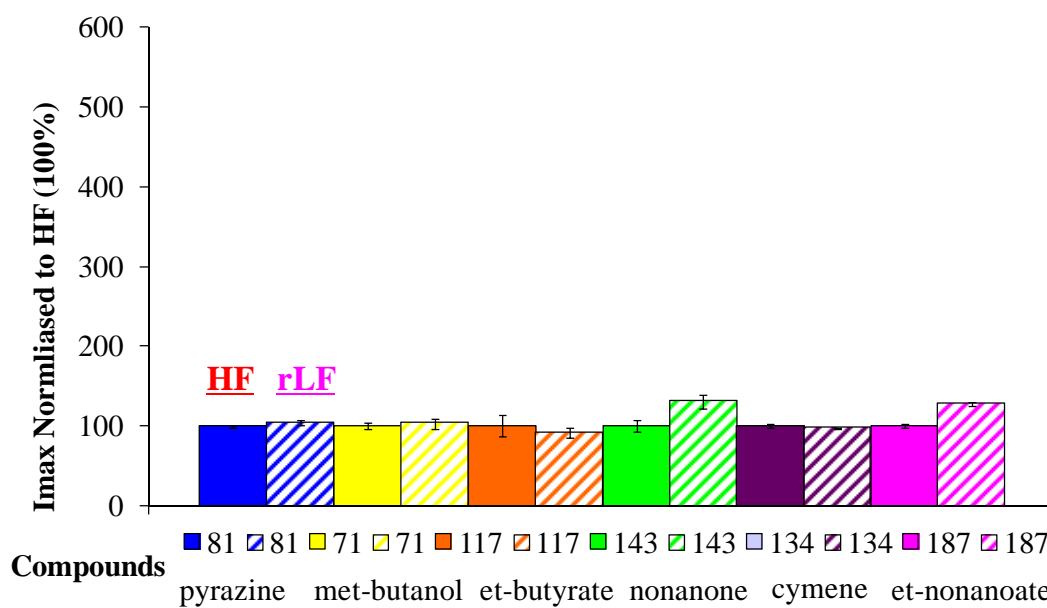


Figure 4.11 In-vivo release of KTP4 flavouring showing the I<sub>max</sub> of high fat yoghurt (HF) as 100% (with filled bar) and normalised I<sub>max</sub> for reformulated low fat yoghurt (rLF) (with striped bar). Different colour represents different compounds, labelled by the APCI-MS ion mass and its name underneath. Error bar represented the standard error for each compound.

In additional to Figure 4.11, ANOVA indicated no significant difference between I<sub>max</sub> of reformulated LF yoghurt and I<sub>max</sub> of target HF yoghurt for all six compounds ( $p > 0.05$ ). Therefore, this model with good prediction of lipid effect on flavour release could be applied to guide flavour reformulation for food products with different fat content. Additional sensory tests were carried out to confirm if the reformulated flavour in LF yoghurt tasted similar to target HF yoghurt with original flavour.

#### 4.2.4 Results of Sensory Validation (Reformulated Yoghurt)

Four commercial flavourings- strawberry (SB), banana (BN), blackcurrant (BC) and coffee (CO) were reformulated from model equation based on their respective

compound Log P values and fat content in LF and HF yoghurt (FC1= 2.7% & FC2 = 11%). The reformulated flavourings (KTPSB, KTPBN, KTPBC, and KTPCO) were applied into LF yoghurt, and compared with related original flavourings in LF yoghurt, which were also the reference samples presented at the first place. Panellists were asked to taste the reference then evaluate the two coded samples (with reformulated and original flavour), and finally choose which of the two matched the reference. The aim was to confirm if the reformulated flavourings in the LF yoghurt could be distinguished from their original flavourings.

Using total number of 36 panellists, the minimum number of correct responses required was 24 in order to conclude that a perceptible difference exists at the significant level  $p < 0.05$  (BS-ISO-10399, 2004). The results of all four flavourings obtained from Fizz are summarised in Table 4.10.

*Table 4.10 Results of duo-trio test for four flavourings in low fat yoghurt with the original and reformulated flavouring for strawberry (SB / KTPSB), banana (BN / KTPBN), blackcurrant (BC / KTPBC) and coffee (CO / KTPCO).*

Test	Answers Taken	Answers Right	Significant Level
<b>SB/ KTPSB</b>	36	19	0.4340
<b>BN / KTPBN</b>	36	24	0.0326*
<b>BC / KTPBC</b>	36	17	0.6911
<b>CO / KTPCO</b>	36	21	0.2025

According to Table 4.10, only reformulated banana flavouring (KTPBN) indicated a significant perceptible difference ( $p < 0.05$ ) from its original flavouring (BN) in low fat yoghurt. The other three reformulated flavourings in low fat yoghurt were not perceived as significantly different from their original flavourings. This might be explained by the flavour difference between these original and reformulated flavourings in the product above their threshold level.

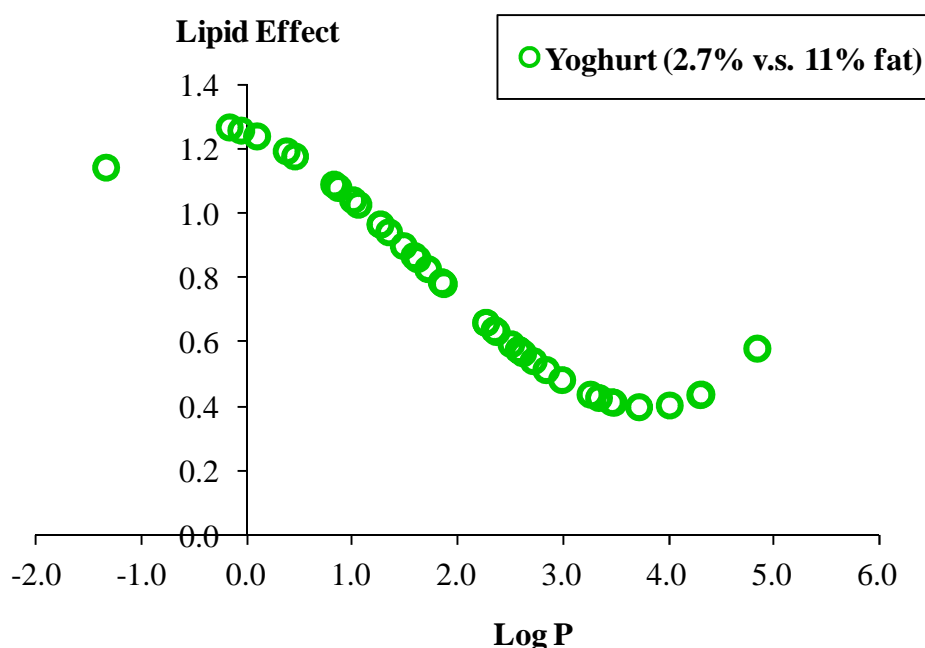
Additionally, using forty commonly used aroma compounds involved in this study, Table 4.11 summarised their estimated Log P values and the lipid effect values calculated from the model (Equation 3.2) based on the fat content in two yoghurt products - 2.7% (FC1) and 11% (FC2).

If the value of lipid effect is close to one, the impact of aroma release difference between low fat and high fat version is minimal. The smaller the lipid effect value, the less of that compound will be released in high fat product comparing with its release in low fat version. As shown in Table 4.11, the aroma release for more hydrophobic compounds like limonene (Log P = 4.83) in low fat yoghurt was predicted to be 0.58 times less than its release in high fat version. Whilst, the less hydrophobic compounds like diacetyl (Log P = -1.34) was predicted to have 1.14 times more released in the reduced fat version.

*Table 4.11 The estimated Log P values of forty aroma compounds with respective Lipid Effects calculated from the modelling equation (FC1= 2.7%, FC2= 11%)*

Compound	Log P	Lipid Effect
diacetyl	-1.34	1.14
acetaldehyde	-0.17	1.27
pyrazine	-0.06	1.26
acetic acid	0.09	1.24
methyl acetate	0.37	1.20
furfuryl alcohol	0.45	1.18
2,5-dimethyl-4-hydroxy-3(2H)-furanone (furanol)	0.82	1.09
ethyl acetate	0.86	1.08
butyric acid	1.00	1.04
vanillin	1.05	1.03
3-methyl butanol	1.26	0.97
amyl alcohol	1.26	0.97
guaiacol	1.34	0.94
hydroxyphenyl-2-butanone	1.48	0.90
trans-2-hexenal	1.58	0.87
hexen-3-ol	1.61	0.86
benzaldehyde	1.71	0.83
hexenoic acid	1.84	0.79
ethyl butyrate	1.85	0.79
2,4-heptadienal	1.86	0.78
ethyl-2-methyl butyrate	2.26	0.66
isoamyl acetate	2.26	0.66
methyl hexanoate	2.34	0.64
methyl cinnamate	2.36	0.63
methyl phenyl carbinyl acetate	2.50	0.60
gamma decalactone	2.57	0.58
2-octenal	2.57	0.58
cis-3-hexenyl acetate	2.61	0.57
trans-2-hexenyl acetate	2.61	0.57
nonanone	2.71	0.54
ethyl hexanoate (ethyl caproate)	2.83	0.52
methyl dihydrojasmonate (hedione)	2.98	0.49
2,4-decadial	3.33	0.43
citral	3.45	0.42
geraniol	3.47	0.41
jasmone	3.71	0.40
para-cymene	4.00	0.41
alpha-ionone	4.29	0.44
ethyl nonanoate	4.30	0.44
limonene	4.83	0.58

Based on the data shown in Table 4.11, Figure 4.12 was used to illustrate the relationship between predicted lipid effect (LE) and compound hydrophobicity (Log P). With increasing Log P values, the lipid effect showed a general trend of reduction from around 1.2 to 0.4 when Log P is around 4. This was a novel approach to demonstrate the effect of lipid in product for a range of compounds at different hydrophobicity.



*Figure 4.12 Calculated lipid effect values from the model equation between low fat (2.7%) and high fat (11%) yoghurt for forty compounds within a range of Log P values*

Consequently, the average Log P value for all aroma compounds in these four flavourings might be a useful indicator: 1.31 (Coffee), 1.64 (Strawberry), 2.15 (Blackcurrant), and 2.44 (Banana). The average Log P for banana flavouring was the highest, and the lipid effect was calculated as 0.61 based on the model, so this flavouring is the most dependent on the impact of fat. For coffee and strawberry flavouring, the lipid effect was calculated as 0.95 and 0.85, so the reformulation of these two flavourings based on the lipid effect may be too small to make a sensorial difference.

However, the reformulated blackcurrant flavouring with lipid effect of 0.69 also indicated no significant sensorial difference, and this might indicate some limitations of this model's application. This blackcurrant flavouring consisted of more than 20 compounds and was the most complex flavour of the four, so it was more challenging for the model to reformulate and for the panellists to evaluate. Therefore, the application of model reformulation may be limited on complex flavourings, and its prediction power should be stronger for more hydrophobic flavourings.

In a further experiment, an orange flavouring (Aromco Ltd) was used with an average Log P value of 3.08. To minimise the acidity and texture variations from commercial yoghurt products, single cream (19.4% fat) diluted with water and 5% sugar was added, to give a final emulsion with 1% or 10% fat. This further study was carried out by Linforth et al. (2010), they applied the model in order to add high- or low-level orange flavouring into respective emulsions both at 0.15%. Firstly, in the triangle test, 12 out of 21 panellists were able to identify differences between the low- and high-flavour formulation in 1% fat system ( $p < 0.05$ ). Following this, the duo-trio test was carried out, 26 out of 40 panellists judged that 1% fat sample with reformulated flavouring was perceived as most similar to the 10% fat sample with original flavouring than the original flavoured 1% fat ( $p < 0.05$ ).

In summary, the modelling reformulation was proven with certain capacity to predict flavour release differences and these differences could be evaluated sensorially in yoghurt products. In addition to yoghurt products, the model was also applied to confectionery products using a range of Aromco's commercial



flavourings. The results of reformulated flavouring not only display better aroma profile, but also reduced cost in use for Aromco's customers. More commercial benefits resulted as the successful reformulation of these flavourings from modelling are discussed in Chapter 8. Other factors that may need to be considered during flavour reformulation are discussed in the next section.

### 4.3 FURTHER DISCUSSION

#### 4.3.1 Other Factors Affecting Flavour Reformulation

##### 4.3.1.1 Source of Log P estimation

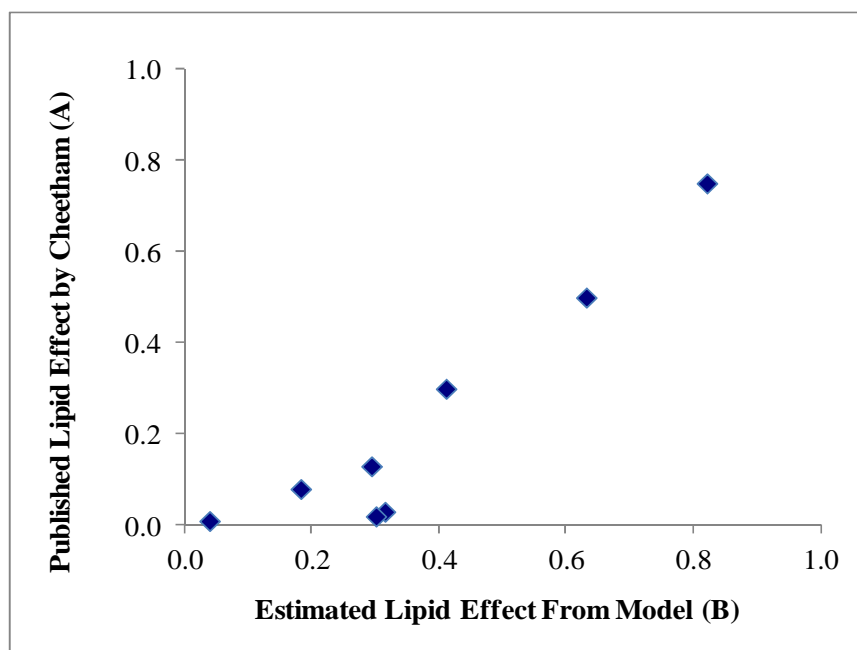
Despite reformulation by either instrumental analysis or model prediction, the Log P was used as an essential indicator for compound hydrophobicity. However, the different methods of Log P estimation could result in variation in model prediction of the lipid effect. Cheetham (2010) published some Log P values used to reformulate vanilla flavouring between high- and low- fat ice creams. Unfortunately, no information was given on how Log P values were calculated from Cheetham, these values were however compared with the estimated results by EPIsuite<sup>TM</sup> (Table 4.12).

*Table 4.12 The Log P values from two sources: (A) published by Cheetham and (B) estimated by EPIsuite<sup>TM</sup>. The Log P difference was calculated as value from method B divided by that from source A for every compound in vanilla flavourings used in ice cream.*

Compound	Log P Estimation		Log P Difference (B/A)
	Cheetham (A)	EPIsuite <sup>TM</sup> (B)	
Vanillin	0.40	1.05	2.63
Phenol	0.89	1.51	1.70
p-Cresol	1.28	2.06	1.61
4-Ethyl guaicol	1.74	2.38	1.37
Eugenol	1.94	2.73	1.41
Ethyl benzoate	2.64	2.32	0.88
Methyl cinnamate	2.79	2.36	0.85
Anethole	3.33	3.39	1.02

Anethole showed similar Log P values between the two sources. Most compounds had higher Log P values estimated by EPIsuite<sup>TM</sup> than those published results by Cheetham. Another two compounds (ethyl benzoate and methyl cinnamate) showed lower Log P values from EPIsuite<sup>TM</sup> than those of Cheetham. Cheetham

also published the reformulation factor for the vanilla flavouring applied to ice cream with 0% fat from the original 15% fat. The predicted lipid effect based on our model (Equation 3.2) was compared with their published results, as shown in Figure 4.13.



*Figure 4.13 Relationship between published lipid effect by Cheetham (A) and estimated lipid effect from model (B) to reformulate vanilla flavouring from 15% fat to 0% fat.*

The lipid effect model generally illustrated good correlation with results from Cheetham for this application. The two outliers were found to be ethyl benzoate and methyl cinnamate, whose Log P values were lower from values from EPIsuite<sup>TM</sup>. This highlights the importance of obtaining the Log P values from the same source as those used to generate the original model (Linforth et al., 2010). Further advances in Log P estimation may produce better quality values for modelling.

#### 4.3.1.2 Nature of aroma compounds

There is some potential for including other modelling terms, such as volatility and the air-water partitioning coefficient ( $K_{aw}$ ) in model development. During previous variation study with 4 panellists eating 9 replicates of either chewy candy or pectin, it was found that the extent of variation was related to the partitioning behaviour of the compounds. The  $K_{aw}$  values can be estimated by EPIsuite<sup>TM</sup> (Section 3.1.1) for six compounds in KTP4 flavouring, and their respective Log  $K_{aw}$  values were calculated, as shown in the parenthesis: pyrazine (-3.92), 3-methyl butanol (-3.27), 2-nonanone (-1.95), ethyl butyrate (-1.77), ethyl nonanoate (-1.20) and p-cymene (-0.32).

The percentage coefficient of variation (CV%) on the I<sub>max</sub> for release during consumption of pectin jelly and chewy candy was calculated for every panellist, and then these values were averaged across the four panellists to produce an average CV% for each compound released from pectin and chewy candy. The relation between these results and Log  $K_{aw}$  is shown in Figure 4.14.

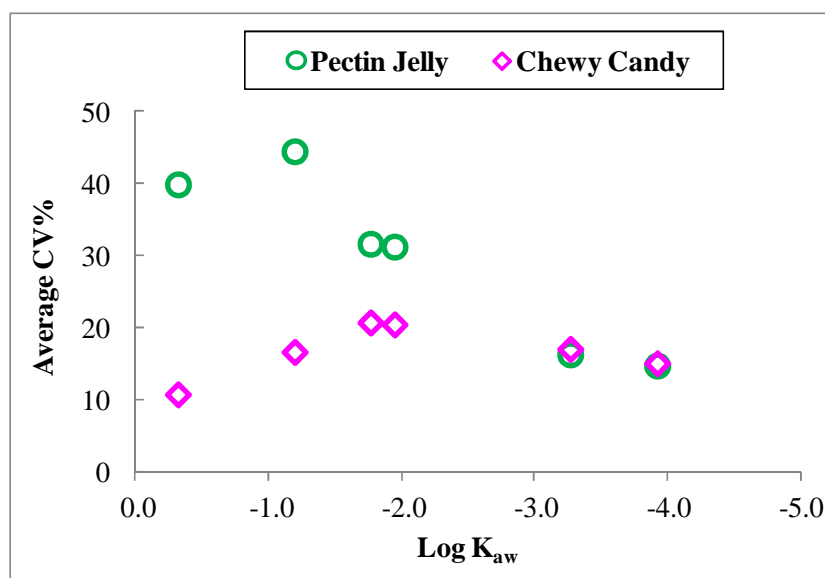


Figure 4.14 Average coefficient of variation percentage (CV%) of the I<sub>max</sub> during consumption of pectin jelly and chewy candy for six compounds with different air-water partition coefficients (Log  $K_{aw}$ ).

According to Figure 4.14, the variation range across all different compounds for chewy candy (CV% range 10-20%) was smaller than pectin jelly (CV% range 15-45%). Pectin jelly showed a greater variation in release for compounds with the highest Log  $K_{aw}$  values (*p*-cymene, -0.32 & ethyl nonanoate, -1.2). Conversely, the compounds with the lowest Log  $K_{aw}$  values (Pyrazine, -3.92 & 3-methyl butanol, -3.27) showed the least variation and overlapped with the variation results of chewy candy.

Linforth et al. (2011) explained the potential reason behind those results. The differences in mass transfer for the compounds released from the pectin gels may be linked to the observed variation differences. Some compounds with high  $K_{aw}$  values produced less than 5% breath aroma concentration compared with their headspace aroma concentration (Linforth et al., 2002). Therefore, the potential for variation with very low levels of aroma release in-vivo is greater than when release is closer to the thermodynamic maximum.

On the other hand, chewy candy did not show the expected differences related to  $K_{aw}$  in aroma release variation during consumption. The lipid in the chewy candy could interact with the more hydrophobic compounds, like *p*-cymene and ethyl nonanoate that have high  $K_{aw}$  values, resulting in lower partition ratios for the chewy sweets compared to the pectin gels. The lower partition coefficient of the chewy candy would increase the efficiency of delivery of volatiles into the breath relative to the thermodynamic maximum, represented by the static partitioning equilibrium state. This, in turn, reduces the potential for variation in release.

Generally, compounds with highest  $K_{aw}$  showed more variation in release than those with low  $K_{aw}$  values. Sample matrix components (such as lipid) have the

potential to reduce the partition coefficient and thereby reduce the variation in volatile release. However, if  $K_{aw}$  was included in the model equation, there may be greater errors associated with its estimation of reformulation due to the errors associated with  $K_{aw}$  estimation. Linforth et al. (2010) stated that the models of Log P are potentially the most researched and robust, and also the most applicable to modelling changes in flavour in-vivo release because they numericise the hydrophobicity of molecules.

#### 4.3.1.3 Differences between food systems

The model prediction for 40 compounds within a range of Log P values is summarised in Figure 4.15, based on the fat difference in one candy group and two yoghurt groups involved in the reformulation study.

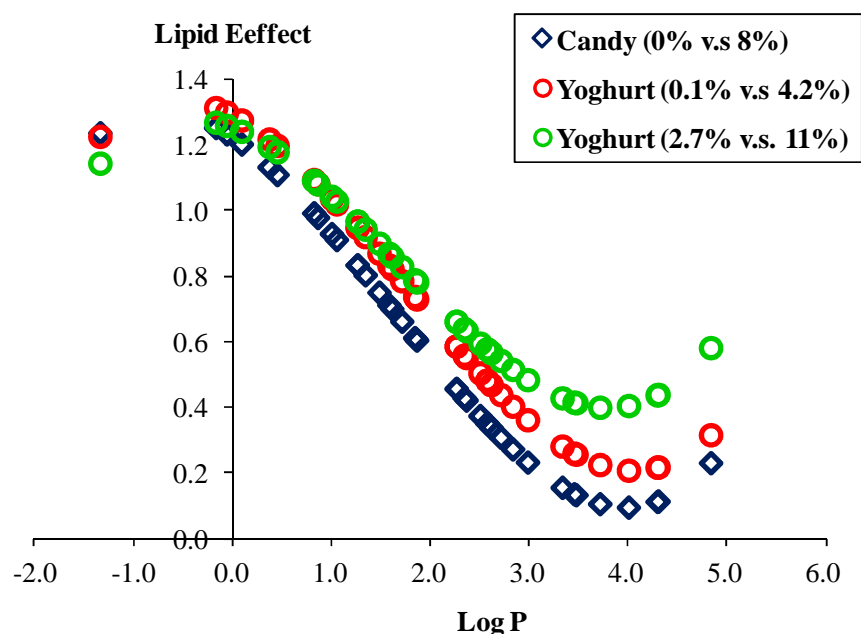


Figure 4.15 Summary of the predicted lipid effect to reformulate 40 compounds in a candy group (pectin jelly v.s. chewy candy) and two yoghurt groups (low fat v.s. high fat): the percentage in the bracket indicated the fat level within the group.

With increasing Log P, a similar trend was shown among the three groups in Figure 4.15. More hydrophobic compounds ( $\text{Log P} > 3.0$ ) showed larger differences in release. These differences were due to fat level differences

consistent with the model equation. Therefore, the fat level difference between the target food and the reformulated food plays an essential role in flavour delivery. Regarding to Figure 4.15, the candy group showed the lower lipid effect values comparing 0% fat with 8% fat matrix, whilst, the yoghurt group with 2.7% and 11% fat illustrated the smallest lipid impact for more hydrophobic compounds.

Besides aroma-fat interactions, protein also plays an important role in flavour studies. The interactions between aroma compounds and protein were reviewed in Section 1.2.3.3. Yoghurt products normally have higher level of proteins (e.g., 4.5% and 5.9% for LF and HF respectively) than candy (0% and 0.4% for pectin jelly and chewy candy respectively). When aroma compounds irreversibly react with proteins, their release from food products will be reduced noticeably. Their interactions with protein could vary depending on the chemical groups, e.g., ketones may bind stronger (2-nonanone) with protein than esters (ethyl butyrate), illustrated by ONeill and Kinsella (1987). Therefore, protein may need to be considered as another factor to add in the model for products with high protein content. However, to add another factor into the model, a series of experiments or literature values would be required. It may also important to gather the data describing the experimental conditions, such as different temperature, pH, etc, including the type of protein to be classified.

Another difference between yoghurt and candy is that yoghurt was made and kept at 3-5 °C, while candies was made at much higher temperatures (110 -130 °C). Thermal processing might alter the flavour release from the final products, so another factor may be required for a specific model for candies or other thermal processed food. Whilst, another model for cold processed food like yoghurt and

ice cream might be modified with a reduced factor. However, the model illustrated the capacity to predict flavour release from different food products, so far, so it has the potential to be directly used for flavour reformulation.

The largest difference between final products of candy and yoghurt is their distinct texture. The candy was solid and hard to chew, while yoghurt was semi-solid and easy to swallow. Gierczynski et al. (2011) comprehensively reviewed the main factors that can influence in-mouth aroma release, focused on yoghurt products. The results suggested that the potential relationship between in-vivo aroma release and aroma perception may be strongly dependent on product (liquid, semi-solid, solid), the inter-individual variability of subjects (mainly chewing behaviour, saliva rate and composition) and their influence on aroma release. Therefore, it was considered, if separate models should be developed for solid, semi-solid and liquid products.

Liquid products (e.g., milk or emulsion) were one of the most common systems used in many experiments since they are homogeneous. Shojaei et al. (2006a) measured the release of ethyl hexanoate from low-fat (LF = 0.1%) and regular-fat (RF = 3.6%) milk using around 90 panellists, and found the maximum breath concentration ( $I_{max}$ ) from LF milk was nearly as twice release that of RF milk. The relative value calculated from the model was 2.35, which was very close to their actual result. Semi-solid products like LF and HF yoghurt used in this study (Section 4.2.3) also showed good correlation between model prediction and actual in-vivo results.

On the other hand, fat in solid food systems may exist in different forms (solid, liquid, or crystalline forms) and there may also be phase separation, so aroma



release could be influenced further. Although the model showed good prediction for confectionery products, a larger data set would be necessary to fully assess the use of this model for solid food systems. Some baked products studied by other researchers were evaluated. Brauss (1999) found the lipid effect of anethole release from high-fat (18%) and low-fat (4%) biscuits was 0.23, and the predicted level from the model was very close at 0.35. Additionally, Dimelow (2004) measured the release of three compounds (anethole, pinene, and carvone) from baked crumb coating with 7% and 0.4% fat, the measured lipid effects (0.24, 0.24, and 0.25) were similar to model predicted values (0.23, 0.20, and 0.30). Therefore, the model could be applied as a guide for flavour reformulation regardless of solid, semi-solid or liquid food systems.

All in all, no model is perfect, but the model does illustrate its ability to predict flavour delivery differences for a range of products with different fat levels. Linforth et al. (2010) published this current model and asked the other researchers to use it with improved terms or additional model factors to develop the model further.

#### 4.3.2 Comparison of Instrumental and Modelling Approaches

The process of reformulating flavours using in-nose aroma release measurements was demonstrated in two types of candy- pectin jelly and chewy candy. The advantages of instrumental approach are: 1) actual in-vivo release profiles between products are collected by direct measurement; 2) it can be applied to a wide range of food applications; 3) it is relative simple and quick to conduct involving five panellists consuming three replicates of each sample; 4) the results

should be accurate and reproducible with high level of replication and careful control of the sources of variations.

The disadvantages of instrumental analysis are: 1) variations between products during manufacturing or flavour addition and variations between panellists due to different physiology or chewing patterns are unavoidable, so experiments are also associated with inherent errors; 2) using average values of release ratio may only satisfy a certain proportion of the population; 3) it can be time and cost consuming to carry out experiments as a series of procedures are needed to follow, from sample preparation, instrument set-up, actual measurement to final data analysis; 4) not all the compounds can be measured because of the limitation of APCI-MS analysis.

Direct measurement becomes more difficult with more complex flavourings when several compounds cannot be detected by APCI-MS due to i) their concentration in the final products was lower than the detection of limits of APCI-MS , ii) they have same molecular weight that cannot be differentiated by APCI-MS ; iii) they are certain compounds like sulphur compounds beyond the sensitivity of APCI-MS . The possible solutions are i) to make another flavourings with compounds at higher concentration (e.g. x10 or x100 more) to find their release ratios between products; ii) to choose indicators compounds with similar properties but which differ in molecular weight, iii) to find alternative compounds that can be measured by APCI-MS or simply apply the release ratio from other measurable compounds with similar Log P values.

To overcome these limitations of measurement approach, an alternative way of flavour reformulation is based on the model established by Linforth et al. (2010).

The predicted values of the model have shown good correlations with the actual measurement results, illustrated by the reformulated KTP4 flavouring in low fat yoghurt. This showed similar release for most compounds when compared with its original flavouring in high fat yoghurt. Furthermore, sensory evaluations using commercial flavourings in yoghurt products were found to be challenging as the average hydrophobicity of the flavouring may need to be considered.

Therefore, the disadvantages of model reformulation are: 1) model may depend on the type of flavourings due to its limitation at extremes Log P values because the middle of the experimental design space is usually the best while there are fewer supporting data at the periphery; 2) predictions from the model might not be always accurate and valid for all the food applications, so both instrumental and sensory analysis are required to confirm its prediction power; 3) other factors or modelling terms may need to be considered to improve its accuracy.

However, the model does appear to have good correlations with actual measurements (see Figure 4.13, Section 4.3.1), and the developed model to reformulate flavourings has the following advantages: 1) it is much faster approach than carrying experiments using the instrumental approach; 2) the predicted values are easily applied for all components when their Log P values are calculated by EPIsuite<sup>TM</sup>; 3) the availability of the model could reformulate flavourings for any two products at different fat content, although other sensory property difference (e.g. texture) caused by reduction of fat should also be considered.

Therefore, the instrumental analysis could provide data to compare with theoretical model, and this model could be a useful guide for flavourists during

flavour reformulation. Regardless of the type of approach applied, it is essential to conduct sensory analysis in order to validate the reformulation results if there is any correction required between sensory perception and release analysis or prediction.

## **PART III FLAVOUR STABILITY**

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### **5 INTRODUCTION**

As numerous factors affect flavour stability in food (Section 1.3.3), it is very challenging for a flavour company to ensure high quality flavour in the end product, especially during long storage periods. During the past twenty years, the instability of citrus flavouring had attracted lots of attention, particularly attempts to stabilise its key component - citral in beverage were reported by several authors (Kimura et al., 1983; Hiramoto et al., 1999; Choi et al., 2009). Vanillin is another very important aroma compound with an aldehyde group and it is also one of the most popular flavour ingredients for food products (Sinha et al., 2008), so vanillin was decided to be the focus component for this study.

Vanillin had been studied comprehensively in dairy products (Graf & De Roos, 1996; Anklam et al., 1997; Gassenmeier, 2003), especially in ice-cream, looking at its interactions with protein and fat. However, little data is available for vanillin stability in bakery products, which is also a major area for its application. Compared with other bakery products such as breads, cakes or pastries, biscuits have relatively long shelf-life (Kilcast & Subramaniam, 2000), and therefore require a more stable flavour preparation. The stability of vanillin within biscuit during storage was chosen to be the main area of study for this work.

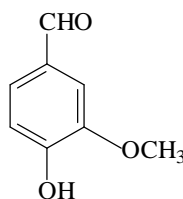
During preparation, vanillin is dissolved in a flavour solvent to make a vanilla flavouring, which is then added into the dough and mixed to enhance dispersal. Although propylene glycol (PG) and triacetin (TA) are two widely used flavour solvents for this application both will interact in different ways with flavour

compounds (see Section 1.3.2). The influence of solvent on flavour release and perception was studied by Potineni (2008) for chewing gums, but no information about the effect of flavour solvent on aroma stability for biscuits can be found.

Biscuits may mean different things to different people, as it can be a generic term including shortcakes, cookies and crackers (Edwards, 2007). Biscuits vary in terms of physical and sensory characteristics, which is dependent on the particular recipe and manufacturing process involved. The biscuit preparation used for this study is short-cake which has a short or crumbly texture as a result of a high fat content that inhibit the formation of protein (gluten) strands (Chevallier et al., 2002). A standard shortcake recipe was modified by Aromco to provide a standardised matrix with a relatively bland base in order to more easily demonstrate the impact of added flavourings. The changes of physical, chemical and sensory attributes in biscuits during storage are reviewed in the Section 5.2.

## 5.1 CHANGES OF VANILLA FLAVOURING

Vanillin is the major component of natural vanilla, which is extracted from the bean or pod of the vanilla orchid. However, natural vanilla can be expensive and contains up to 200 other organic compounds (Sinha et al., 2008). Synthetic vanillin with the same molecular structure as its natural form was used in this study for purity and repeatability.



*Figure 5.1 Molecular structure of vanillin*

As shown in Figure 5.1, vanillin (4-hydroxy-3-meth-oxybenzaldehyde) has both aldehyde and phenolic groups, so it can undergo several types of reactions. The following three types of reactions may lead to its organoleptic loss during storage: i) flavour solvent effect; ii) oxidation; iii) Schiff base formation; iv) physical interactions with starch and proteins.

#### 5.1.1 Flavour Solvent Effect

Solid vanillin is a crystalline material that has limited solubility. A flavour solvent is usually required to dissolve vanillin but it will also interact chemically with vanillin forming flavour-solvent interactions. For example, the use of PG as solvent may react with vanillin to form PG-acetal (Elmore et al., 2011). However, analysis of PG-acetal was not covered in the scope of this PhD research.

Compared with the more hydrophilic solvent PG, TA as a more lipophilic solvent makes vanilla flavouring easier to disperse throughout the oily dough matrix. When the matrix is then further processed, the changes may lead to structural differences in the dough and alteration of the final product quality attributes. There is no published research comparing the effect of solvent choice on bakery products, but Potineni (2008) reported significant differences in chewing gum: chewing gum formulated with TA was softer and was perceived to have a higher flavour intensity than the one formulated with PG. Therefore, it is interesting to compare the effects on the solvent choice on the stability of vanillin in aged biscuits and to study the effect of these two solvents on the biscuit structure.

#### 5.1.2 Vanillin Oxidation

Vanillin oxidation is one of the many reactions reported, and vanillic acid could be formed by either enzymic (Anklam et al., 1997) or thermal oxidation (Mourtzinou et al., 2009). In yoghurt products, the oxidation of vanillin to vanillic

acid can take place during the processing of milk by the action of enzymes that are present in fresh and pasteurised milk (Graf & De Roos, 1996) and is shown to be dependent on the local microchemistry (Anklam et al., 1997).

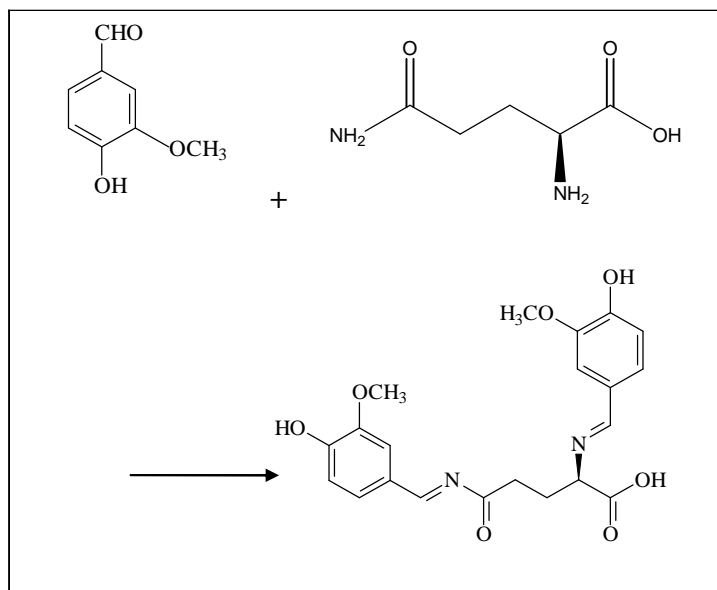
Chemical oxidation of vanillin was reported by Mourtzinou et al. (2009) when pure vanillin was heated under isothermal or non-isothermal conditions. They noticed that only 9.4% vanillin had been oxidised to vanillic acid over the temperature range 131- 258 °C. However, whether vanillic acid is formed in bakery products or not is unknown, so it is essential to check its existence during this experiment.

#### 5.1.3 Schiff Base Formation

A considerable amount of vanillin loss during storage can be explained by Schiff base formation, i.e., an aldehyde or keto group of volatiles (such as vanillin) bind to amino side groups of protein (such as glutamine), as illustrated in Figure 5.2.

The literature suggests that the interaction of vanillin and protein via the Schiff base arrangement could be irreversible (Feeney et al., 1975) or reversible (Kim & Min, 1989). Many studies have shown that the Schiff base formation is temperature dependent: Ho et al. (1988) observed acceleration of the reactivity at higher temperatures (ranged from 80, 90 to 100 °C); whilst Ge and Lee (1997) showed that the rate of Schiff base formation was slower at lower temperature (comparing 40 °C with 54 °C). Generally, the reaction rates were accelerated as the temperature increased based on kinetic studies of vanillin interaction with amino acids in the model system (Chobpattana et al., 2000). Therefore, it is possible that the increased storage temperature results increase the Schiff base formation and thus further reduces the vanillin level in biscuits.





*Figure 5.2 Schiff base formation of vanillin and glutamine*

Additionally, Graf and De Roos (1996) suggested that the Schiff base formation between vanillin and proteins can be accelerated in ice cream by lowering the fat content. It was assumed that the oil phase can dissolve vanillin and protect it further away from aqueous medium in which the Schiff base condensation can take place. Therefore, a similar hypothesis for our experiments is proposed, the lipophilic solvent TA would result in more vanillin being retained in the biscuit after storage compared to the more hydrophilic solvent PG.

#### 5.1.4 Physical Interactions with Starch and Proteins

Most low-molecular weight organic molecules, particularly aldehydes such as vanillin, are known to adsorb to polymeric structures in food (Graf & De Roos, 1996). Reineccius (2006a) stated that starch and proteins in biscuits offer substantial opportunity for flavour binding both in the baking process as well as during storage. Losses during baking are reduced by using a lipid-soluble flavouring (such as TA) compared with a more water-soluble flavouring (such as PG) as volatiles will have reduced vapour pressure in an oil solvent providing better retention during this process.

Vanillin forms strong phenolic hydrogen bonds with protein and also helical inclusion complexes with linear starches. The binding of volatiles to starch or protein can be measured analytically (Hau et al., 1996), but the addition of starch and proteins may have no functional effect on the odour perception of vanillin (Reiners et al., 2000) because transiently bonded flavours may be released by mastication and swallowing of the food (Graf & De Roos, 1996). As a result, it is necessary to measure the change of vanillin levels in biscuits after storage by instrumental analysis and also to evaluate the changes in perceived vanilla intensity involved by sensory analysis during biscuit storage studies.

## **5.2 CHANGES OF BISCUIT DURING STORAGE**

One aim of the study was to investigate the change of vanillin in biscuit during storage, but it is also necessary to understand and track other key characteristic changes to biscuits under different storage conditions. The instrumental methods in shelf-life testing can monitor physical and chemical changes in biscuits during storage, and sensory tests are essential to reflect if these changes can be perceived (Kilcast & Subramaniam, 2000).

### **5.2.1 Physical Changes**

The most commonly used physical test for biscuit quality is texture analysis. Biscuits typically absorb moisture during storage which leads to hydration of the matrix and a change of texture in the form of sogginess or loss of crispness. This can be prevented by careful handling and proper packaging. Textural differences are normally evaluated by texture analysis with carefully chosen probes, cross-head speeds, sample positions and alignment, to maximise the correlation of analytical results with sensory measurements.

### 5.2.2 Chemical Changes

The two most important chemical changes associated with biscuits are non-enzymatic browning during biscuit baking and lipid oxidation during biscuit storage, both of which need to be evaluated.

#### 5.2.2.1 Non-enzymatic browning

The flavour chemistry of baked products and of biscuits in particular is complex with many volatiles being present at low concentrations. For instance, 5-hydroxymethyl-furfural (HMF, as shown in Figure 5.3, i) can be formed as an intermediate in the Maillard reaction (Morales et al., 1997) or from direct thermal degradation of sugars through caramelisation (Kroh, 1994). The rate of formation in biscuit is dependent on the process temperature, type of sugar, pH and moisture content (Ait Ameer et al., 2008). HMF can then degrade into 2-furfuraldehyde (Figure 5.3, ii) at trace level by decarboxylation (Kroh, 1994). In addition, both compounds can be lost subsequently due to their volatility and their ability to bind with other compounds (Capuano & Fogliano, 2011).

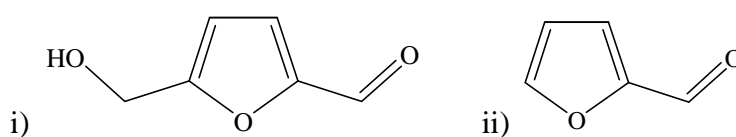


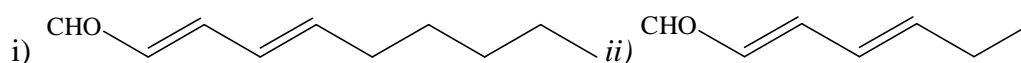
Figure 5.3 Chemical structure of i) HMF and ii) 2-furfuraldehyde

Both caramelisation and Maillard reactions produce brown polymeric compounds that contribute to the surface colour of the baked biscuits. Browning at the surface has previously been used as a reaction indicator to control other baking processes such as bread (Ramirez-Jimenez et al., 2001), so colour measurement is also predicted to be a useful indicator of biscuit baking intensity in this study.

#### 5.2.2.2 Lipid oxidation

Lipids are one of the least stable bulk components of food, as lipids can be degraded by oxidation chemistry resulting in the development of rancidity depending off-notes; this is dependent on the degree of unsaturation of the lipids.

The rate of lipid oxidation development is influenced by several factors. Presence of oxygen in the vicinity of food and increase of temperature play critical roles in influencing the rate of reaction. Lipid oxidation will also lead to the generation of a wide range of hydroperoxides which then degrade to form secondary products. The types of volatile compounds produced during the oxidation of edible oils are influenced by the composition of the intermediate hydroperoxides formed which in turn is dependent on the fatty acid profile. For example, the autoxidation of polyunsaturated fatty acids, such as linolenic acid (Ullrich & Grosch, 1987), produces volatile secondary oxidation products including 2,4-decadienal and 2,4-heptadienal (Figure 5.4).



*Figure 5.4 Chemical structure of i) 2,4-decadienal and ii) 2,4-heptadienal*

The 2,4-alkadienals might be considered to contribute desirable aroma of a variety of freshly prepared foods, but they are also found in foods with oxidised or stale flavours and they can contribute a deep-fried fat odour or fried oily aroma (Saison et al., 2009). They have low threshold values as 0.04 – 0.3 ppm from oxidised edible oils, reported by Frankel (1985) and their concentrations should be minimised in most food materials.

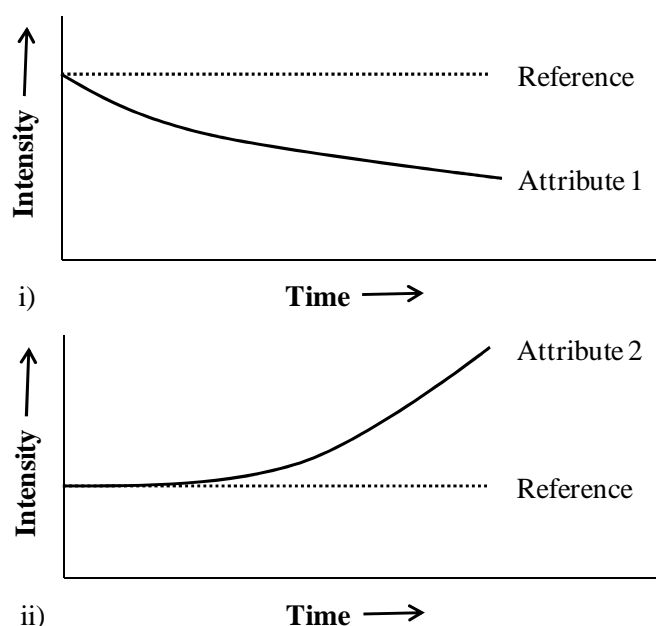
In addition, photochemical reactions may generate volatile compounds with positive or negative effects on flavor quality (Chen & Ho, 1998). Shahidi (2000) stated that breakdown of lipids under photo-oxidative conditions produces an array of products, many of which are odour-active and may contribute to off-flavor development in both raw and processed foods. Milk (Sattar et al., 1975) and other lipid containing products are susceptible to light induced deterioration. This reaction has been studied in soybean oil (Neff et al., 1993), olive oil (Kiritsakis & Dugan, 1985) and butter (Veberg et al., 2007). However, this is not the focus of the study, so it is essential to control the exposure of sample to light during the biscuit storage tests.

### 5.2.3 Sensory Changes

The evaluation of aroma and texture stability can be directly or indirectly related to the sensory assessment. Sensory evaluation by consumer panels usually gives a good estimate of the overall quality state of a food. Heenan et al. (2009) identified the sensory properties that contribute to the perceived freshness of different baked products, including breads, biscuits and cakes. The crunchy texture, buttery flavour and malty odour were illustrated as useful terms to distinguish similarities and differences in the descriptions of freshness for the three product types.

Kilcast (2000) reviewed various sensory test procedures that generate information on whether changes are occurring, the nature of the changes that are occurring and the magnitude of the changes. One of the most commonly used sensory tests is to measure the attribute change in product quality, with a reference sample at a fixed level of change. This is illustrated in Figure 5.5 for two sensory attribute with i) decreasing intensity and ii) increasing intensity. These two attributes in this study

were assumed to indicate changes of vanilla flavour (Attribute 1) and changes of off-flavour development from lipid oxidation (Attribute 2).



*Figure 5.5 Illustrative changes in sensory attribute with time at i) decreasing intensity ii) increasing intensity compared to the reference samples*

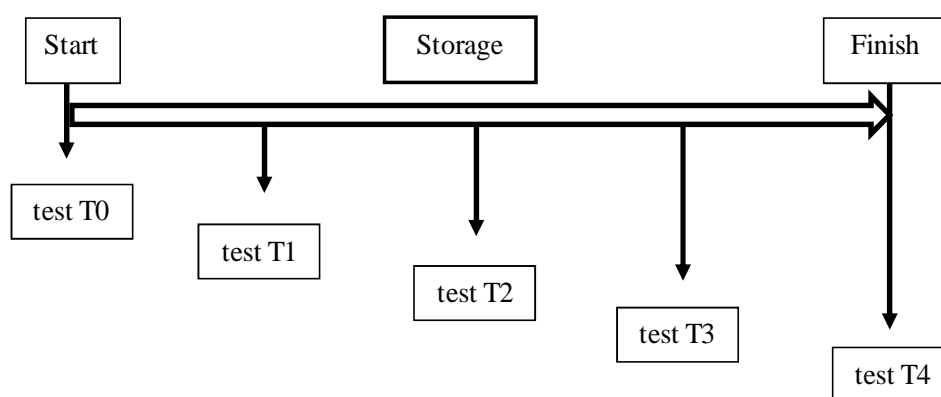
### 5.3 ACCELERATED SHELF-LIFE TEST

In order to obtain necessary information to determine the shelf-life of a food product in a relatively short time, food industry attempted to use accelerated shelf-life (ASL) test to shorten the process and explore the key test parameters (Mizrahi, 2000). The basic premise of the test is assumed that accelerated deterioration can be achieved by raising the storage temperature so a correlation of accelerated shelf life and real shelf life can be defined (Labuza & Schmidl, 1985).

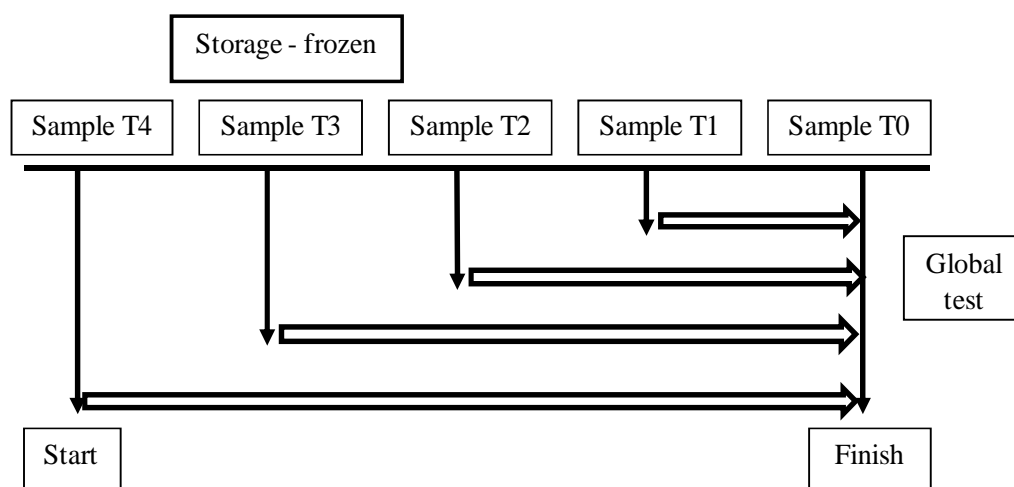
Mizrahi (2000) reviewed various kinetic models by the ASL test to predict the actual shelf-life of various food products. However, in the complex food system, additional changes may take place at elevated temperatures compared with normal storage conditions. Take biscuit for instance, the increased temperature may induce phase changes from the melting of fats and changes in solvent properties.

Furthermore, one mathematic model might not be applied to several types of deterioration reactions (Calligaris et al., 2007). As a result, the overall effect on biscuit quality is often not predictable, and any ASL model can lead to either under- or over-estimated shelf-life predictions.

Instead of using ASL test to predict the shelf-life of biscuits, the purpose of this study was to evaluate product stability based on the data obtained by the ASL test in a relative shorter period (e.g. 8 weeks, the true shelf-life of the biscuit is about 12 months). Two options of experimental design were demonstrated by Kilcast and Subramaniam (2000), as shown in Figure 5.6 and Figure 5.7.



*Figure 5.6 The partially staggered design for shelf-life testing*



*Figure 5.7 The drawn sample design for shelf-life testing*

Figure 5.6 illustrated the partially staggered design (Gacula, 1975), which is the most commonly operated type of test. All products on test are stored at time zero and samples are taken off for testing at time intervals. The advantage of this design is that data related to shelf-life are generated at intervals and build up to give a moving picture of change in deterioration. The disadvantage is that errors could happen during sample preparation and the instrument shifting at different test periods.

According to Figure 5.7, a large batch is held under frozen conditions. Samples are then removed at appropriate intervals and stored under the test conditions. The advantage of this design is that all samples are analysed at the end of shelf-life test, and the limitation is that prior knowledge of expected shelf-life is required to set up the trials.

It is also possible to combine the merits of both experimental designs in order to develop the most appropriate method for biscuit shelf-life test in this study. In both design types, the shelf-life tests are susceptible to fluctuations in production quality, so in all cases strict quality management and a preliminary variation analysis is vital to ensure the consistency of products that are used for the ASL tests.



## 5.4 RESEARCH OBJECTIVES

The aim of the study was to increase understanding to help maintain flavour quality in the food product. The main focus of the study was on vanillin stability within stored biscuits, whilst also comparing two flavour solvents (PG and TA). In addition to vanillin, other changes in biscuit quality were investigated by instrument, sensory and structure analysis. The research objectives are defined as follows:

### 5.4.1 Objectives of Instrumental Analysis

- (1) To select biscuits with a minimum variation after baking as measured by colour, texture and aroma analysis and to preliminarily evaluate the effect of flavour solvent by these three types of analysis.
- (2) To explain the impact of storage on the changes of vanillin level on the ASL stored biscuits.
- (3) To explain the impact of storage on the changes of other aroma compounds generated from non-enzymatic browning and lipid oxidation for the ASL stored biscuits.
- (4) To explain the impact of storage on the changes of texture in the ASL stored biscuit.
- (5) To explain the impact of flavour solvent statistically ( $p < 0.05$ ) between two flavour solvents -PG and TA for the ASL stored biscuits.
- (6) To compare the ASL test with the normal shelf-life test on biscuit texture and aroma changes.

#### 5.4.2 Objectives of Sensory Analysis

- (1) To identify if the changes in the ASL stored biscuits are significantly perceptible by consumers through paired comparison tests ( $p < 0.05$ ).
- (2) To compare the ASL test with the normal shelf-life test on biscuit sensory changes.
- (3) To measure the detectable difference level of the vanillin in fresh biscuits to validate numerically the scale of any sensory differences.

#### 5.4.3 Objectives of Structure Analysis

- (1) To measure and compare and contrast the three dimensional micro-structure of PG and TA biscuits by X-ray CT scanning.
- (2) To evaluate the relative spatial distribution of the added aroma (vanillin) and of the process aroma (HMF) inside biscuits.
- (3) To compare the relative spatial distribution of vanillin and HMF with the three dimensional micro-structure of the biscuit as measured by X-ray CT scanning.

## PART III FLAVOUR STABILITY

### 6 MATERIALS AND METHODS

Biscuits were made from two types of vanilla flavouring using either PG or TA as the flavour solvent. The schematic diagram shown in Figure 6.1 summarises the methodology applied for the flavour stability study.

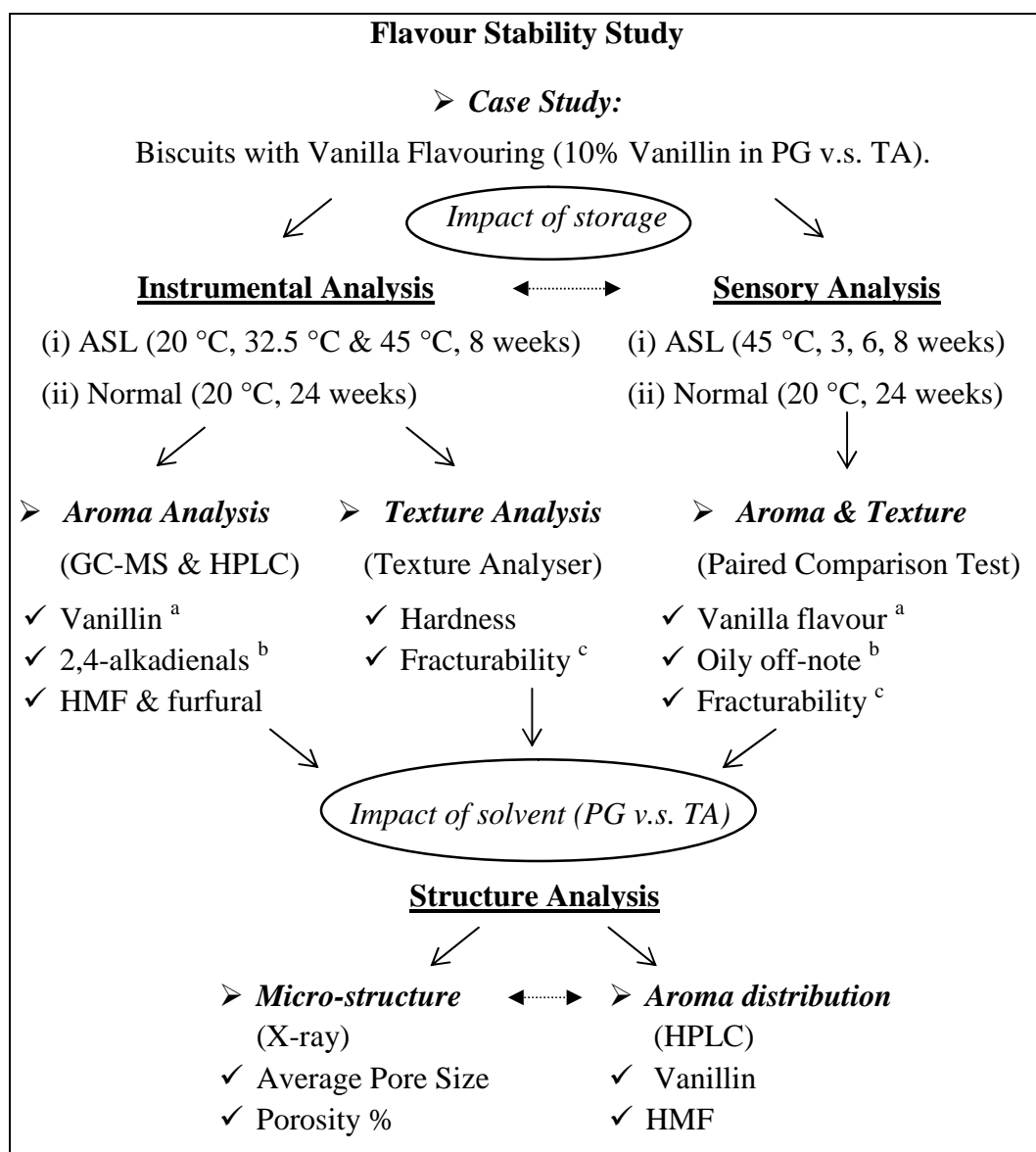


Figure 6.1 Schematic diagram of methodology applied in flavour stability study using instrumental and sensory analysis plus structure analysis. ASL: accelerated shelf-life test; superscripted letter a, b, c: sensory attributes associated to instrumental analysis.

Since the changes of biscuit quality at the ASL test might be different from these at normal shelf-life storage, the impact of storage was evaluated and compared by instrumental and sensory analysis in terms of aroma and texture changes. The aroma analysis was carried out by both GC-MS and HPLC measurement for identification and quantification of vanillin, 2,4-alkadienals, HMF and furfural. A texture analyser was used to measure hardness and fracturability. Sensory evaluations were conducted by paired comparison tests and the sensory attributes selected were correlated with instrumental results as vanilla flavour, oily off-note and fracturability.

X-ray Computer Tomography was used to evaluate the impact of solvent choice on the three dimensional microstructure of the biscuit and to correlate this to aroma generation and vanillin loss. Frisullo et al. (2010) previously applied this technique on different types of biscuits and breadsticks, it was proposed in this study that a combination of aroma chemistry and knowledge of the spatial microstructure of the biscuit may help to explain solvent effects on the stability of quality attributes during ageing. In addition, the distribution for vanillin and HMF at selected locations in PG and TA biscuits was compared at an analytical level. The structure difference between PG and TA biscuits might help explain the previous solvent effect on aroma and texture stability.

Prior to all the detailed experiments, a preliminary variation analysis study was conducted to evaluate the distribution of the colour, texture and aroma in biscuits that were all baked within one tray. This study was designed to pre-screen baking locations so reproducible samples could be produced for analytical testing and shelf life evaluation. It was also designed to investigate if the use of flavour and

the type of solvent have any significant impact on biscuit colour, texture and aroma before storage. Other factors including product composition, processing parameters, packaging, and environmental factors were carefully controlled for all biscuits.

## **6.1 FLAVOURINGS & CHEMICALS**

### **6.1.1 Vanilla Flavouring**

Food-grade vanillin, propylene glycol (PG) and triacetin (TA) were supplied by Aromco Ltd (Nuthampstead, UK). Two simple vanilla flavours were made by mixing vanillin (10% w/w) with PG or TA as the flavour solvent. Both flavourings were made on the day of application, the standard application dosage for both flavourings was 0.2% w/w in the biscuit dough (i.e. 200 ppm of vanillin was added initially).

### **6.1.2 Other Chemicals**

Internal standard was chosen as 3-heptanone ( $\geq 98\%$ , Acros Organics, New Jersey, USA) for GC-MS analysis and acetovanillone ( $\geq 98\%$ , SAFC Supply Solutions, St. Louis, USA) for HPLC analysis. Methanol (HPLC Grade  $\geq 99.9\%$ ) was ordered from Fisher Scientific UK Ltd, Loughborough, UK. Remaining chemicals were purchased from Sigma Aldrich, UK: 5-hydroxymethyl-furfural (HMF,  $\geq 99\%$ ), 2-furaldehyde (furfural, 99%), vanillic acid (97%), 2,4-decadienal (85%) and 2,4-heptadienal ( $> 88\%$ ).

#### *6.1.2.1 Standard solutions for GC-MS analysis*

The standard solution was prepared in methanol for GC-MS measurement including vanillin (10  $\mu\text{g/ml}$ ), 3-heptanone (10  $\mu\text{g/ml}$ ), furfural (4.1  $\mu\text{g/ml}$ ), HMF

(5 µg/ml), 2,4-decadienal (5 µg/ml) and 2,4-heptadienal (5 µg/ml). The concentration for all compounds in this standard solution was defined as 100% for calibration curve purposes. Triplicate samples were made for each concentration level (50%, 100% and 200%).

#### *6.1.2.2 Standard solutions for HPLC analysis*

Similar to the previous section on the standard solution preparation for GC-MS analysis, three standard solutions for HPLC analysis were also prepared at three concentration levels (50%, 100%, 200%), including vanillin (5, 10, 20 µg/ml), acetovanillone (5, 10, 20 µg/ml), vanillic acid (5, 10, 20 µg/ml), furfural (2.05, 4.1, 8.2 µg/ml) and HMF (2.5, 5, 10 µg/ml). Triplicate samples were prepared at every concentration level.

## **6.2 BISCUIT PREPARATION & STORAGE**

### **6.2.1 Materials and Methods for Biscuit Manufacturing**

All the ingredients to make a standard biscuits dough are detailed in Table 6.1. Shortening ‘Crown NH AV Shortening’ was supplied by Cardowan Creameries Ltd (Glasgow, UK), the biscuit flour was sourced from Rank Hovis (High Wycombe, UK) and flavouring was sourced from Aromco Ltd (Nuthampstead, UK). All other ingredients were supplied at food grade by C Holland & Sons Ltd (Royston, UK).

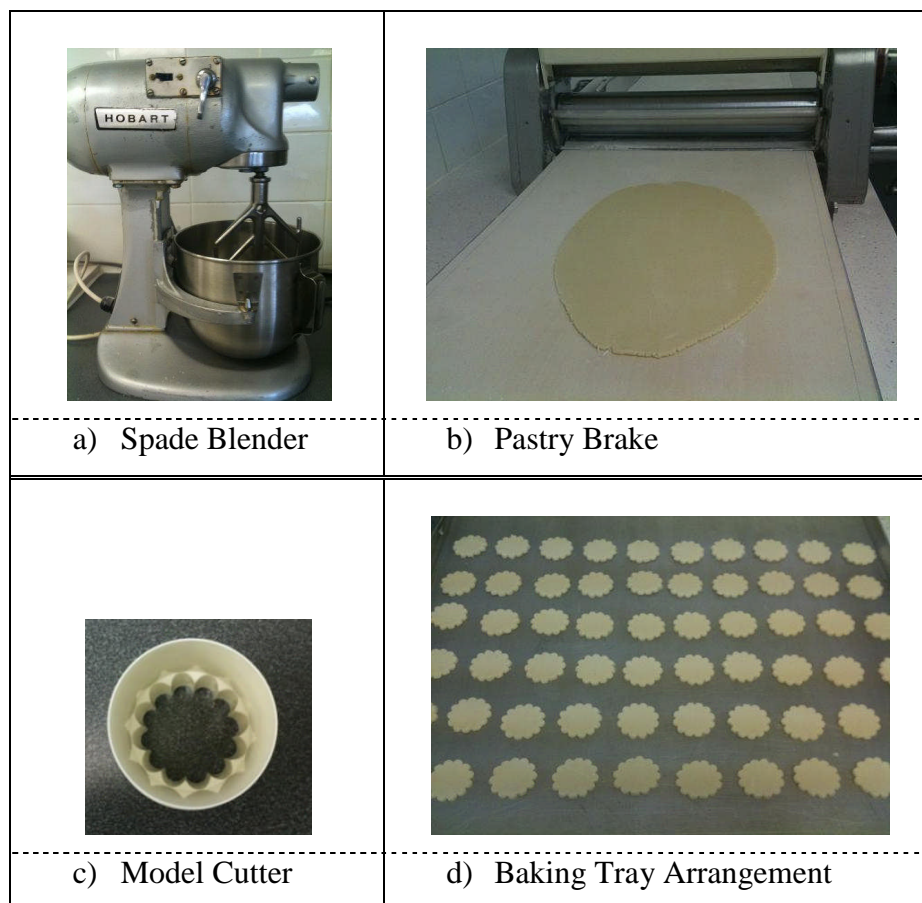
Shortening, as palm oil and rapeseed oil mixture, contains 37% saturated fat, 46% mono-unsaturated fat, 16% poly-unsaturated fat, less than 1% trans-fatty acid and maximum 0.1% free fatty acid.

*Table 6.1. Ingredients and composition for standard dough*

No.	Ingredient	g/100g
1	Shortening	15
2	Icing Sugar	15
3	Invert Sugar	2
4	Skimmed Milk Powder	0.3
5	Salt	0.3
6	Lecithin	0.3
7	Sodium Bicarbonate	0.14
8	Ammonium Bicarbonate	0.03
9	Water	11
10	Biscuit Flour	56
11	Flavouring (if required)	0.02

The ingredients from No.1 to No.6 (as shown in Table 6.1) were weighed and blended by a spade blender (Hobart, Windsor, UK, as shown in Figure 6.2 a). A fat base was then formed after 2 min of continuous mixing of these ingredients. A water base was prepared by dissolving the Ingredients No.7 and No. 8 in cold tap water.

Standard dough was made by gradually mixing flour into the fat base then added the water base into the fat base by a spade blender (Figure 6.2a). The dough was cut into half by weight, both PG and TA flavourings were then added into the respective dough by 2 min blending until the dough was smooth. Each dough preparation was rolled to 4 mm thickness using a Pastry Brake (Seewer Rondon, Burgdorf, Switzerland, Figure 6.2. b). A model cutter (36 mm diameter, round with fluted edge, Figure 6.2. c) was used to shape individual biscuits. The cut biscuit pieces were then positioned with equal separating distances on the baking tray (Figure 6.2. d).



*Figure 6.2. Dough preparation using a) spade blender, b) pastry brake, c) model cutter, d) baking tray arrangement*

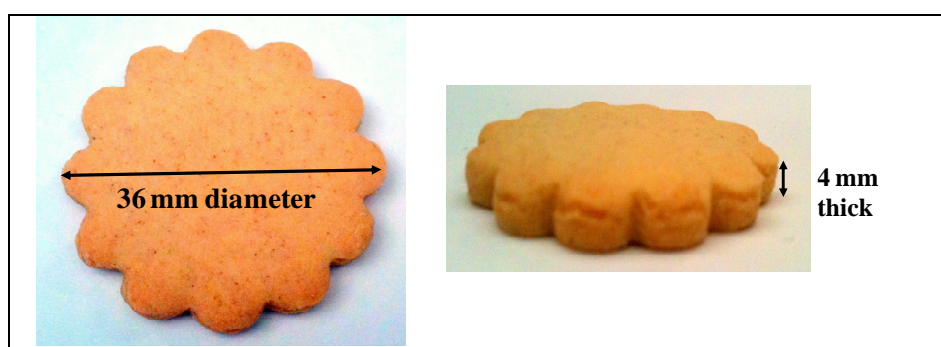
The tray was placed on the top layer of a Deck Oven (Sveba-dahlen, Fristad, Sweden), as shown in Figure 6.3. The biscuits were baked at 230 °C for 8 min on the top layer, and were then dried on the bottom layer of the oven for 3 min at 100 °C. Finally, the baking tray was removed from the oven to allow the biscuits to cool for 10 min at room temperature (25 °C). The biscuits were then carefully packed and stored in sealed aluminium bags with a minimum headspace within the bag.





*Figure 6.3 Deck oven with top layer for baking at 230 °C for 8 min and bottom layer for drying at 100 °C for 3 min*

The biscuit was made as a one-bite size (illustrated Figure 6.4) so that the same amount of flavour could be released for different panellists when the whole piece was eaten during sensory tests.



*Figure 6.4. Demonstration of biscuit shape and size*

#### 6.2.2 Biscuits Prepared for Preliminary Study on Baking Variation

In the preliminary studies, three types of dough were made, as illustrated in Figure 6.5: 'A' blank samples with no flavour solvent added, 'B' samples made with 0.2% PG added, and 'C' samples with 0.2% TA added. No vanillin was used in this study as the focus was to evaluate variations in the biscuits during baking and to compare if the solvent would have any effect on biscuit properties.

Following the standard method of mixing, 40 pieces of each type were made and labelled (A1-40, B1-40, C1-40) and placed randomly on a tray to be baked at the same time. As shown in Figure 6.5, the values of X and Y axis represented the columns (X1-10) and rows of the tray (Y1-12). The randomisation was achieved by 'Rand' function in the Excel (Microsoft Office, 2007) for individual columns from X1 to X10.

Y12	C1	B5	A9	B13	A17	C21	C25	C29	B33	A37
Y11	A1	C5	B9	C13	C17	B21	C26	B29	C33	B37
Y10	C2	A5	A10	B14	C18	B22	C27	C30	A33	C37
Y9	A2	A6	C9	A13	B17	C22	B25	B30	B34	A38
Y8	B1	A7	A11	C14	A18	B23	A25	A29	A34	B38
Y7	B2	B6	C10	C15	A19	A21	A26	C31	B35	C38
Y6	A3	C6	A12	C16	B18	A22	B26	A30	A35	C39
Y5	C3	A8	C11	A14	C19	A23	B27	A31	B36	A39
Y4	A4	C7	B10	A15	B19	C23	B28	B31	C34	A40
Y3	B3	C8	C12	B15	A20	C24	A27	C32	C35	B39
Y2	C4	B7	B11	A16	B20	A24	A28	A32	A36	C40
Y1	B4	B8	B12	B16	C20	B24	C28	B32	C36	B40
	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10

Figure 6.5. Three types of samples (A-blank, B- PG biscuit, C- TA biscuit) arranged randomly in the baking tray (X, Y axis)

After baking and cooling, all labelled biscuits were packed in non-permeable aluminium bags at ambient temperature, which were then analysed within a week for colour, texture and aroma measurement.

### 6.2.3 Biscuits Prepared for Storage Tests

Since biscuits at the edge of the tray were expected to have large variations in baking intensity, they were discarded in the storage studies to minimise analytical variation. One batch of the dough was made and split into two so that half had

vanillin-PG flavouring and another half had vanillin-TA flavouring. The pieces of the dough were cut and positioned in alternative rows in the baking tray with equal separating distances to reduce baking variations.

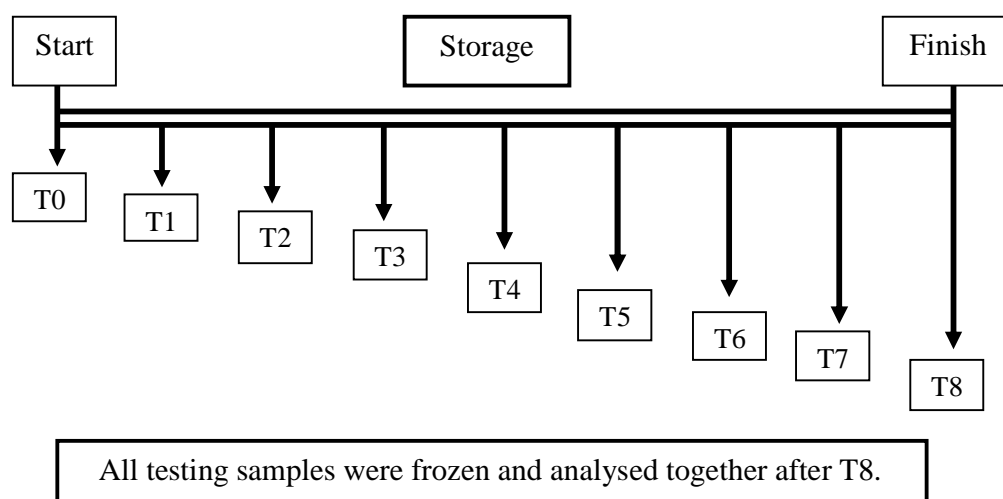
After baking and cooling, all biscuits were stored in non-permeable aluminium bags under normal atmospheric conditions. The use of this type of packaging was to minimise the moisture intake from the environment and control the photo-oxidation reaction induced by light.

#### *6.2.3.1 Samples for the ASL storage*

##### *(A) Experimental design of the ASL test*

The ASL test was carried out in various conditions that may be encountered in the product distribution chain and in the domestic storage environment. In this study, three storage temperatures were selected as 20, 32.5 and 45 °C. In order to determine the end point of the ASL test, biscuit samples were stored previously at 45 °C for 4 and 8 weeks, it was noticed that the product property changed significantly after 8 weeks storage from the fresh samples due to the generation of an oily off-note. Therefore, 8 week storage was determined as the end point of the ASL test.

Comparing previous shelf-life designs (Figure 5.6 and Figure 5.7), the design for this study was adapted (Figure 6.6) with three main stages: i) putting all products on test at time zero; ii) taking off the samples for testing at timed intervals; iii) freezing these samples till the end of the test to minimise sample preparation errors and instrumental shift.



*Figure 6.6 Experimental design for ASL test at time interval from T0 to T8 representing storage week from 0 to 8*

The samples for instrumental analysis covered every storage week at all three storage temperatures. For sensory analysis, due to the large number of biscuits required and severe logistical issues, biscuits stored at 45 °C were used after 3, 6, and 8 weeks storage. These stored biscuits were compared respectively to controlled samples that were kept frozen since T0.

To ensure that deep freezing did not induce any noticeable changes in the biscuits, trials were conducted before the test to evaluate the impact of a single free-thaw cycle. Five panellists attended the triangle test and no panellists could distinguish the freeze-thawed biscuits with the standard product.

### ***(B) Samples for ASL test by instrumental analysis***

Biscuits were baked and stored after cooling in non-permeable aluminium bags, controlled samples were stored at -80 °C and the remaining sample sets were stored at 20 °C, 32.5 °C or 45 °C in scientific ovens (Sanyo Scientific Oven, Loughborough, UK). Triplicate samples were removed weekly from each storage condition and stored in non-permeable aluminium bags at -80 °C. All samples

from all storage treatments and from all time points were tested together in a randomised order.

### *(C) Samples for ASL test by sensory analysis*

Biscuits for sensory analysis were made from same batches as instrument analysis. Half of them were controlled samples stored as at -80 °C, another half were stored at 45 °C in the laboratory oven (Sanyo Scientific Oven, Loughborough, UK) for 3, 6, and 8 weeks. Three sessions of sensory tests were carried out for these stored biscuits after 3, 6, 8 weeks, and the controlled samples were removed from the freezer four hours before each test to allow them to thaw at room temperature.

#### *6.2.3.2 Samples for the normal storage test*

Extra batches of PG and TA biscuits were made with half of them stored at -80 °C as controlled samples and the other half stored at 20 °C (Sanyo Scientific Oven). After 24 weeks of storage, four stored and four controlled biscuits for either PG or TA samples were analysed by the Texture Analyser and GC-MS. The sensory tests for these stored biscuits and controlled samples were conducted at week 24.

### *6.2.4 Biscuits Prepared for Additional Tests*

#### *6.2.4.1 Samples for detectable difference test of vanillin in biscuits*

Four types of biscuits were made with four vanillin-TA flavourings (V0-V3), which differ in the amount of vanillin dissolved in TA, as shown in Table 6.2.

*Table 6.2 Four types of vanillin-TA flavourings used in the vanillin detectable difference test*

<b>Type of Flavouring</b>	<b>Vanillin% in the Flavouring</b>	<b>Vanillin (g) / TA (g)</b>
V0 Flavouring	10% Vanillin (Original %)	10 g / 100 g
V1 Flavouring	9% Vanillin (10% Reduction)	9 g / 100 g
V2 Flavouring	8% Vanillin (20% Reduction)	8 g / 100 g
V3 Flavouring	7% Vanillin (30% Reduction)	7 g / 100 g

All flavourings were prepared in TA one day before baking, and they were all added at 0.2 % w/w into the biscuit dough. They were baked in the same tray to reduce batch-to-batch variation, and after cooling, they were kept in aluminium bags for less than one week before the test.

Four replicated biscuits made from every type of flavouring were randomly selected, extracted and analysed by GC-MS. The rest of the biscuits made from V1, V2 and V3 flavourings were compared respectively to the standard biscuits made with V0 flavouring in the sensory tests (Section 6.4.3).

#### *6.2.4.2 Samples for biscuit structure analysis*

The standard PG and TA biscuits were made in this studying with the standard flavouring (10% vanillin-PG or 10% vanillin-TA) at the standard dosage (0.2%). Both types of biscuits were baked in the same tray following standard process, and they were then kept in two aluminium bags till the test, which was carried out after three days.

### **6.3 INSTRUMENT ANALYSIS**

#### **6.3.1 Colour Measurement**

The colour measurement spectrophotometer ColorQuest® XE from HunterLab Ltd (Reston, Virginia, USA) was used (Figure 6.7.a). The measurement is based

on CIE  $L^*a^*b^*$  scale (Figure 6.7.b). Hunter (1958) explained the scale: the L axis from top to bottom with the maximum value of 100 represents white and the minimum of zero represents black; positive a is red and negative is green; whilst positive b is yellow and negative is blue.

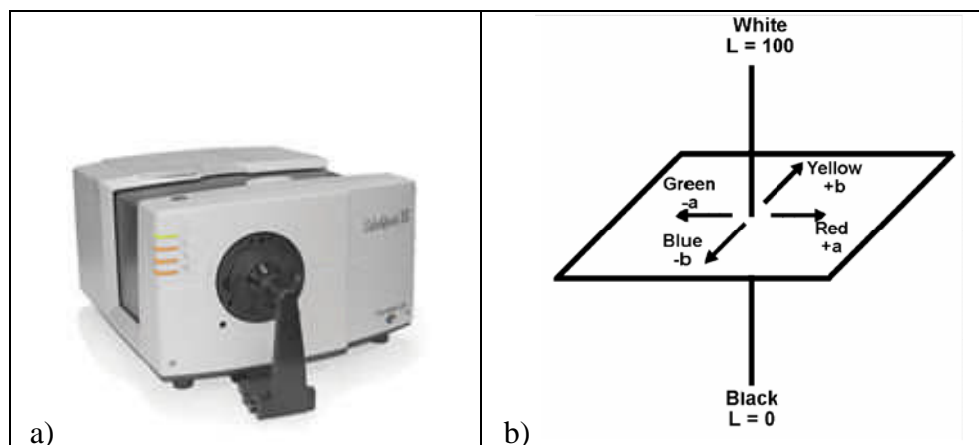


Figure 6.7. a) Colour measurement spectrophotometer and b) CIE  $L^*a^*b^*$  scale

The CIE (Commision Internationale de L'Eclairage, Austria) has codified the different types of white light sources and called them 'illuminants'. The illuminant observer used in our experiment was  $D_{65}$  and represented the daylight. The instrument was standardised on the light trap using the white tile. After calibration, the intact biscuit sample was placed over the reflectance port and held in place using the sample clamp to measure its surface colour. The edge of biscuits may have non-uniform characteristics, so centred locations were measured to minimise errors. After the first colour reading, the sample was rotated  $90^\circ$  to read again, and then another  $90^\circ$  for a third reading. The average from these three colour readings for a single sample was chosen to represent its colour. The averaging multiple readings with rotation between readings minimized measurement variation associated with directionality. The results of L, a, and b were recorded for all biscuit samples.

### 6.3.2 Texture Measurement

The texture analyser TA XT (Stable Micro Systems Ltd, Surrey, UK) was used to measure the force in compression using a heavy duty platform (HDP/90). As commonly used equipment for biscuit texture analysis (Burseg et al., 2007; Pareyt et al., 2009), a 3-point bending rig (HDP/3PB) is shown in Figure 6.8 .



*Figure 6.8 Texture Analyser fitted with a heavy duty platform and a 3-point bending rig involving a probe and two plates with a defined gap at 17.5 mm*

As illustrated in Figure 6.8, the distance in the inner gap between two plates was 17.5 mm and the upper blade linked to the probe moved vertically with 3.25 mm from either side of the plate. The biscuit was placed on the top of two the plates centrally. Pre-test speed was 1.0 mm/s, test speed was 3.0 mm/s and post-test speed was 10.0 mm/s. The trigger type was selected at ‘auto’ mode with the trigger force of 10 g. The maximum force exerted is defined as ‘Hardness (g)’ and the distance to the point of break is termed by the resistance of the sample to bend - ‘Fracturability (mm)’. As illustrated in Figure 6.9, fracturability is obtained by multiplying the breaking time (~0.07) by the test rate (3 mm/s), i.e., fracturability was approximately 0.21 mm, whilst the hardness was around 1600 g for this particular biscuit.



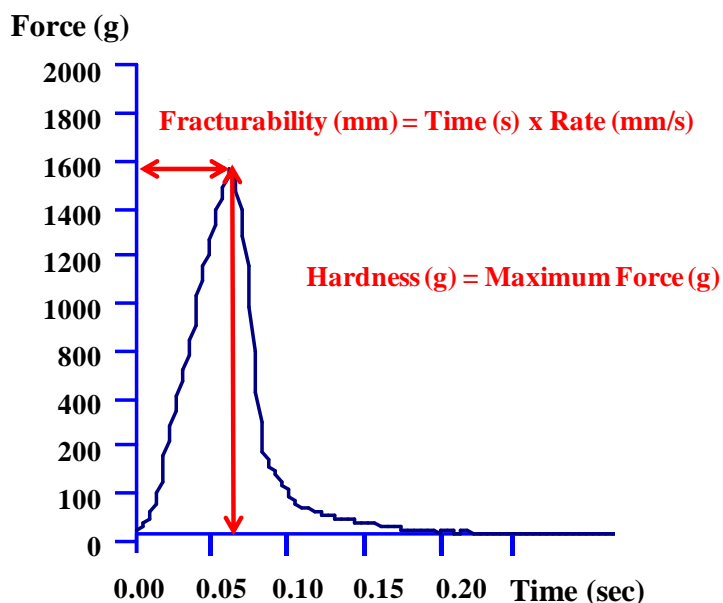


Figure 6.9 Illustration of result graph obtained from Texture Analyser to measure the hardness (g) and fracturability (mm) of one biscuit

### 6.3.3 Aroma Measurement

#### 6.3.3.1 Sample extraction

The intact biscuit (3 g) was ground and 1 g sample was weighed for extraction. Every biscuit sample was extracted with 10 ml methanol, and 100  $\mu$ l of the internal standard was added prior to extraction. The internal standard (IS) consisted of 3-heptanone (123  $\mu$ l) and acetovanillone (100 mg) in 100 ml methanol. The mixed samples were placed on a roller mixer (Thermo Scientific, Tube roller Spiramix 10) to roll side by side for 30 min, and then centrifuged at 1300 RCF for 20 min at 5 °C (Thermo CR3i Multifunction Centrifuge, KeyWrite-DTM). The upper solvent layer was isolated and 1 ml of the extract was filtered (Nylon Syringe-Filter 4 mm 0.4  $\mu$ m) into 2 ml amber vials, capped with Teflon coated lids and analysed by GC-MS and HPLC as appropriate using measurement methods defined in section 6.3.3.2 and 6.3.3.3.

To develop a standard mixing time for efficient extraction, several mixing times (1, 5, 10, 15, 20, 30 and 60 min) were used to define the standard extraction time when biscuits were mixed with methanol. The peak areas of vanillin extracted from biscuits at different mixing times are shown in Figure 6.10. The extracted vanillin level increased with the increased extraction time.

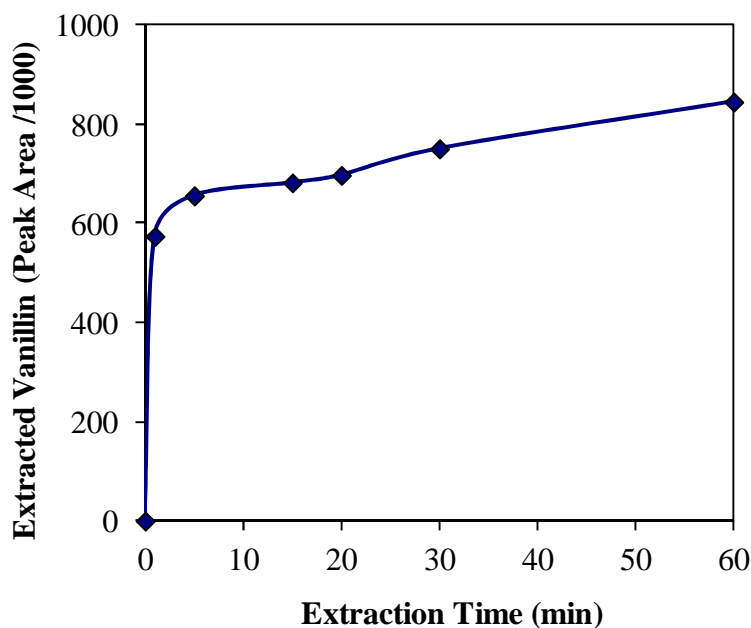


Figure 6.10. Peak area /1000 of vanillin extracted from biscuits at different extraction time (1, 5, 10, 15, 20, 30 and 60 min)

Due to the large number of samples that would be involved in later storage tests, it is essential to prove that a 30 min mixing time is sufficient to extract vanillin from the samples. An additional test was carried out to check the vanillin extraction efficiency using 30 min mixing time. Triplicate biscuits (a, b, c) were extracted with 10 ml methanol for 30 min mixing twice. The peak area for vanillin detected after the first extraction (PA1) was compared with its peak area after the second extraction (PA2). The extraction efficiency (EE) for vanillin could be calculated from Equation 6.1:

$$EE = (PA1 - PA2) / PA1 \quad (\text{Equation 6.1})$$

The vanillin extraction efficiency calculated from the three samples was 93%, 94% and 95%, so the average extraction efficiency was  $94\% \pm 1\%$ . This level illustrated that the standard 30 min extraction should be sufficient to extract the vanillin in biscuits, so 30 min was set as the standard mixing time.

#### 6.3.3.2 GC-MS analysis

Separation and detection of the aroma compounds of interest was achieved with splitless direct injection (1  $\mu$ L) using a Trace GC Ultra (Thermo Scientific, Manchester, UK) coupled with a DSQII mass spectrometer (Thermo Scientific). Separations were performed on the ZB-Wax column - length 30 m, inner diameter 0.25 mm, and film thickness 1  $\mu$ m (Capillary GC Column, Zebron, Phenomenex Inc., Macclesfield, UK).

The initial temperature of the oven was set at 40 °C for 1 min, it was then increased 8 °C per min to 250 °C and held at 250 °C for 6 min. This programme was adapted from Mourtzinis et al. (2009) for identification of vanillin by GC-MS. After several modifications on the temperature and the time of heating and holding, this method was chosen to be the standard because the peak of vanillin was clearly identified and was represented by a clearly defined sharp peak shape.

Single-ion monitoring was used to detect the aromas at specific ions ( $m/z$ ) and retention times (min) as summarised in Table 6.3. Retention time and mass spectra of detected compounds was confirmed by the use of authentic standards. The peak areas were obtained from peak integration with library identification confirmation using 'Xcalibur' software (Thermo Scientific, UK). The concentrations for the compounds of interest are calculated using response factors (peak area of compound of interest/peak area of internal standard).

Table 6.3: GC-MS detected compounds with selected ions and retention time

Profile	Retention Time (min)	Selected Ion (m/z)	Detected Compound
Internal Standard	8.48	114	3-heptanone
Added Flavouring	30.05	152	vanillin
	16.34	45	propylene glycol
	23.59	103	triacetin
Generated Aroma	14.41	96	furfural
	29.54	126	5-hydroxy-methyl-furfural
	20.19	81	2,4-decadienal
	15.04	81	2,4-heptadienal

Based on triplicate standard solutions at 50%, 100% and 200% levels of concentration (as detailed in section 6.1.2.1) a calibration curve of GC-MS analysis was drawn (Figure 6.11) with the standard deviation between replicate samples illustrated as error bars. Regarding all five compounds, the squared correlation coefficient ( $R^2$ ) was higher than 0.91. This calibration result indicated that GC-MS could provide reproducible results.

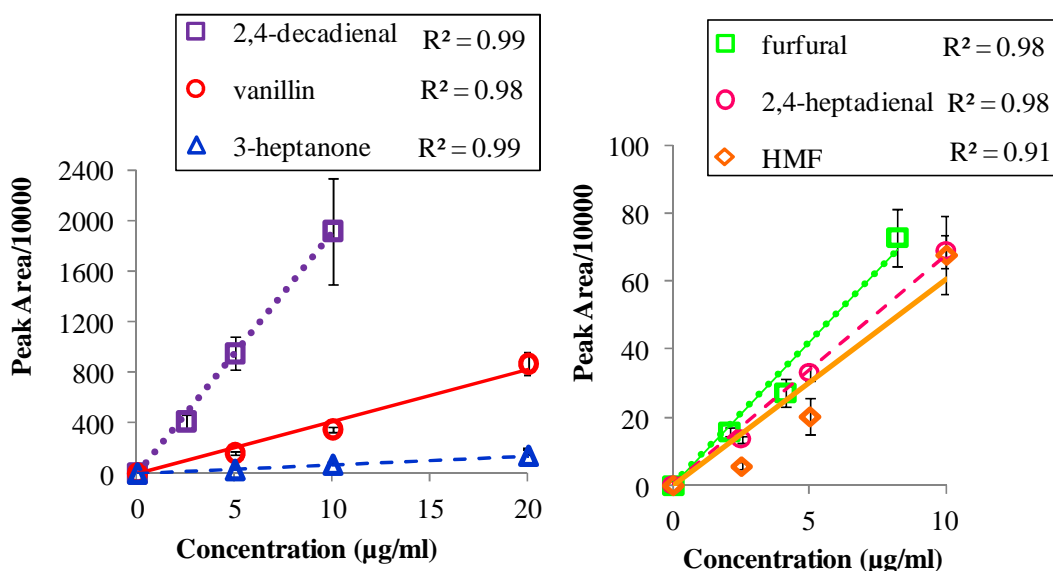


Figure 6.11 GC-MS calibration curve illustrates the compound concentration ( $\mu\text{g/ml}$ ) against its respective peak area /10000 at the maximum scale of 2400 for 2,4-decadienal, vanillin and 3-heptanone, and at the maximum scale of 100 for furfural, HMF and 2,4-heptadienal. Error bars based on the standard deviations between replicated samples and the linear trend lines with their squared correlation coefficient ( $R^2$ ) were also displayed.

#### 6.3.3.3 HPLC analysis

Authentic standards of furfural, 5-hydroxy-methyl-furfural, vanillic acid, vanillin, and acetovanillone were clearly separated as defined peaks at retention times 5.11, 8.46, 11.68, 15.00, 17.93 min respectively by HPLC (Alliance<sup>®</sup> Waters 2095, Waters Corporation, Massachusetts, USA) fitted with by Photodiode Array Detector (PDA, Waters 996). Compounds were measured at 270 nm. Column C18 Techsphere with 250 x 4.6 mm from Thermo Scientific (Manchester, UK) was used.

Compared to the HPLC method used by Ait Ameer et al. (2006) for HMF analysis in cookies, instrumental settings for this study were developed as follows: injection volume 10 µl, flow rate 1 ml per min, gradient elution with water (1 % acetic acid) and methanol (ramped from 20 % to 50 % over 30 min then to 100 % over 1 min and held for 2 min). The chromatography data was analysed by Millenium<sup>32</sup> software (Waters, USA). The retention time for the detected compound was summarised in Table 6.4.

*Table 6.4: HPLC detected compounds at respective retention time*

Profile	Retention Time (min)	Detected Compound
Generated Aroma	5.11	5-hydroxy-methyl-furfural
	8.46	furfural
	11.68	vanillic acid
Added Flavouring	15.00	vanillin
Internal Standard	17.93	acetovanillone

Calibration curves by HPLC-PDA analysis was obtained (Figure 6.12) by measuring standard solutions (prepared in 6.1.2.2) with error bars based on the standard deviations from triplicate samples. The results of  $R^2$  for all compounds

shown in Figure 6.12 exceeded 0.980, which indicated the high repeatability of HPLC measurement.

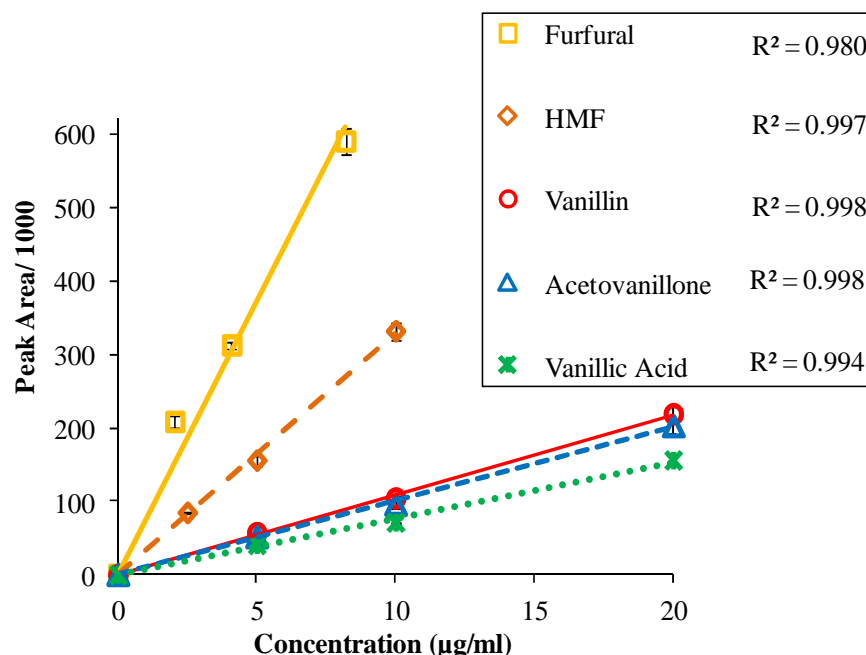


Figure 6.12: HPLC calibration curve for standard solutions (including furfural, HMF, vanillin, acetovanillone and vanillic acid) displayed with error bars as standard deviations from triplicate samples and linear trend lines with squared correlation coefficient ( $R^2$ )

The known concentration of the standard solution (100% level) was used to calculate the concentration of interest compounds from the biscuit extract. The literature (Braithwaite & Smith, 1996) derived equations were modified in this study to calculate vanillin concentration in biscuits. Using acetovanillone (100 µg) as the internal standard, the extracted vanillin amount (µg) can be obtained by comparing the peak areas (PA) of these two compounds in the biscuit sample and in the standard solution (SS) respectively (Equation 6.2).

Extracted Vanillin (ug) =

$$\frac{PA \text{ (Vanillin in the sample)}}{PA \text{ (Vanillin in the SS)}} \times \frac{PA \text{ (Acetovanillone in the SS)}}{PA \text{ (Acetovanillone in the sample)}} \times 100 \mu\text{g}$$

(Equation 6.2)

The calculated amount in  $\mu\text{g}$  could be transferred into ppm for 1 g of sample used. Additionally, other compounds like HMF and furfural can be calculated using a similar approach.

#### 6.3.4 Data Analysis for Instrumental Analysis Results

The contour plots for colour, texture and aroma data were generated from Minitab 16 Software (Minitab Inc., Pennsylvania, USA). The bar charts comparing the average results for three types of biscuits were obtained from Chart Builder function of SPSS16.00 (SPSS Inc., Chicago, USA).

The results of L, a, b from colour measurement were analysed by Multivariate Analysis of Variance (MANOVA) in SPSS16.0 using either Location X or Y as the fixed factor and following Post Hoc test for these locations by Tukey's multiple comparison tests (MCT) ( $p < 0.05$ ). Similarly MANOVA-Tukey's MCT was applied to analyse the results of texture and aroma measurement referring to any significant effect of solvent and different locations on the tray ( $p < 0.05$ ).

For data collected during the ASL test at different storage temperatures and times, the line graphs were generated for both PG and TA biscuits by Chart Builder of SPSS16 and displayed with standard error bars. The results obtained for all the biscuits during texture and aroma measurement were analysed by MANOVA-Tukey's MCT for storage time and storage temperature. ANOVA was applied to indicate any significant difference observed between PG and TA biscuits ( $p < 0.05$ ).

After the normal shelf-life storage, MANOVA was applied to compare the results of all aroma compounds (vanillin, HMF, 2,4-decadienal and 2,4-heptadienal) with the solvent type and storage conditions as fixed factors.

## **6.4 SENSORY ANALYSIS**

### **6.4.1 Paired Comparison Test for the ASL Storage**

In order to define the key attributes that might change significantly during biscuit storage, a preliminary trial on biscuits stored for 7 weeks at 45 °C was conducted and three attributes were identified as being the most likely to change on storage: brittleness (fracturability), vanilla flavour and an oily off-note.

A paired comparison test (BS-ISO-5495, 2005) was performed by 30 biscuit consumers (aged at 21-31 years old, 14 male, 16 female). Prior to the test, the definition and test method for each attribute was clarified to the panellists. Since fracturability may be difficult for panellists to understand, a more 'brittle' biscuit was described as being more easily broken down on the first bite, which is related to fracturability measurement. Panellists were instructed to bite the middle of the biscuit with their front teeth and then spit out any biscuit entering the oral cavity. Vanilla flavour was demonstrated by asking panellists to smell the difference between a jar of blank biscuits and a jar of vanilla flavoured biscuits. The lid for a bottle of vanillin was also available for sniffing if the panellist was still unsure of the vanilla flavour. To evaluate the vanilla flavour biscuits, the panellists were asked to eat the whole biscuit so the portion size was controlled. The final attribute, on oily off-note, was illustrated by allowing panellists to smell some trials biscuits previously stored at 45 °C for 12 weeks, which had an oily off-note.



Each panellist assessed each group of samples (PG biscuits and TA biscuits) to give a total of 6 paired comparison tests. Half of the panellists carried out paired comparisons for PG biscuits first and the other half for TA biscuits according to a randomised balanced design. A five minute break was taken by the panellists after the first 3 evaluations. They were asked to cleanse their palate with water (Evian, Danone, France) between samples. All biscuits were presented in standard 30 ml medicine cups labelled with random 3 digit codes. These tests were repeated in three sessions with biscuits after 3, 6 and 8 weeks of storage (at 45 °C).

All tests were designed by 'Fizz' software and carried out in the sensory booth at  $18 \pm 1$  °C under red light, to minimise the effect of any variation in biscuit colour. Data was collected using Fizz sensory software.

#### 6.4.2 Paired Comparison Test for the Normal Shelf-life Storage

In order to evaluate the results of the ASL test, biscuits stored at normal conditions (24 weeks, 20 °C) were compared with the controlled samples (24 weeks, -80 °C). Three paired comparison tests were carried out for three attributes –brittleness (fracturability), vanilla flavour and oily off-note in both PG and TA biscuits. Similar procedures to Section 6.4.1 were applied for 30 biscuit consumers, and the data was collected by Fizz.

#### 6.4.3 Detectable Difference Test for Vanillin in Fresh Biscuits

Since the main objective of this study focused on the vanillin changes in biscuits during storage, it was necessary to confirm if the reduced vanillin level tasted significantly different from its original level in fresh biscuits.

Three triangle tests (BS-ISO-4120, 2004) were conducted for three groups of biscuits (a, b, c) prepared in section 6.2.4.1: Group a) standard V0 biscuits with original vanillin level and V1 biscuits with 10% vanillin reduction level; Group b) standard V0 biscuits and V2 biscuit with 20% vanillin reduction; Group c) standard V0 biscuits and V3 biscuits with 30% vanillin reduction.

Thirty panellists (University students, age 21 – 32) attended for these tests, and the order of the tests was balanced. A two-minute break was given to the panellists between each test, and water (Evian, France) was provided to cleanse their palate. All biscuits were presented in standard 30 ml medicine cups labelled with random 3 digit codes generated by Fizz software.

#### 6.4.4 Data Analysis for Sensory Analysis Results

All the sensory data were collected and analysed by Fizz software to calculate the values of  $\alpha$  risk, if the results were significantly,  $\alpha$  risk would be less than 0.05. The BS-ISO-5495 (2005) and BS-ISO-4120 (2004) was used as standard method for paired-comparison test and triangle test respectively.

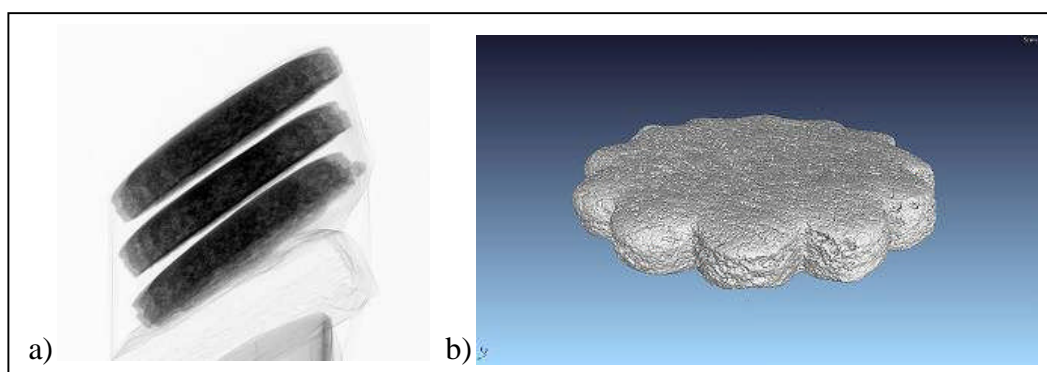
### 6.5 STRUCTURE ANALYSIS

#### 6.5.1 X-Ray Computed Tomography

Computed Tomography (CT) is a non-destructive technique for visualising solid interiors, and X-ray attenuation is a function of density of the material (Babin et al., 2007). By the sample rotation, a series of 2-D X-ray images can be obtained, which can then be rendered into a 3-D image and allow accurate reconstruction of the internal microstructure (Kerckhofs et al., 2008). In this study, the microstructure of three PG biscuits and three TA biscuits was analysed through X-

ray CT scan with 3D metrology by Phenoix Nanotom NF180 CT System (GE Sensing & Inspection Technologies GmbH, Wunstorf, Germany).

Three replicated biscuits were fixed together in the position shown in Figure 6.13 a), in order to be rotated and scanned as one stack. With the electron acceleration energy set at 80 kV and current at 180  $\mu$ A, the angular projects obtained was 1440, which was the number of images acquired over the 360° rotation. The scan resolution was 22.5  $\mu$ m, and Figure 6.13 b) illustrated the 3D image of a biscuit after reconstruction.

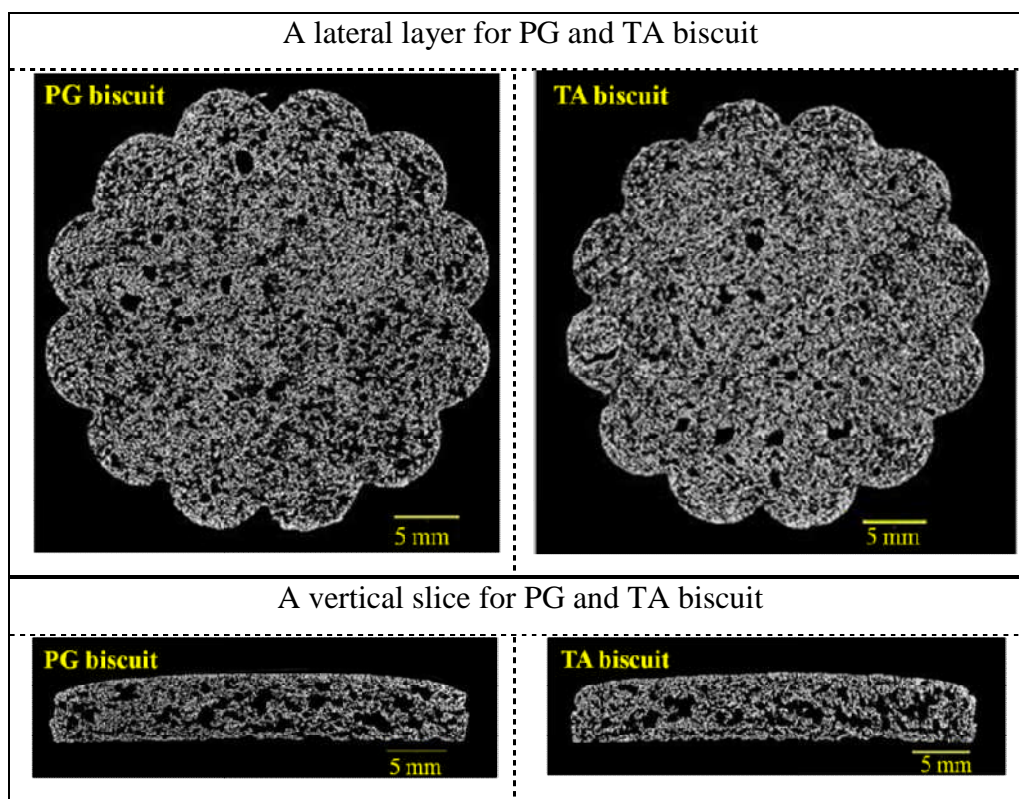


*Figure 6.13 a) Arrangement of three biscuits to be scanned together; b) 3D image of one biscuit after reconstruction*

All images were analysed by 'Image J' processing software version 1.44 (public domain Java analysis programme, developed by National Institute of Health, Maryland, US) <http://rsb.info.nih.gov/ij/>. This software was used by several authors (Chaunier et al., 2007; Pareyt et al., 2009) to obtain quantitative data from digital images of food.

The images obtained at RGB colour and the colour threshold was adjusted as black and white with default function by 'Image J' analysis. Without any physical destruction, a cross section view of the lateral layer and the vertical slice for both PG and TA biscuit was obtained from CT scan, as demonstrated in Figure 6.14.

The white areas shown were the biscuit matrix, and the black areas inside the biscuits indicated pores.

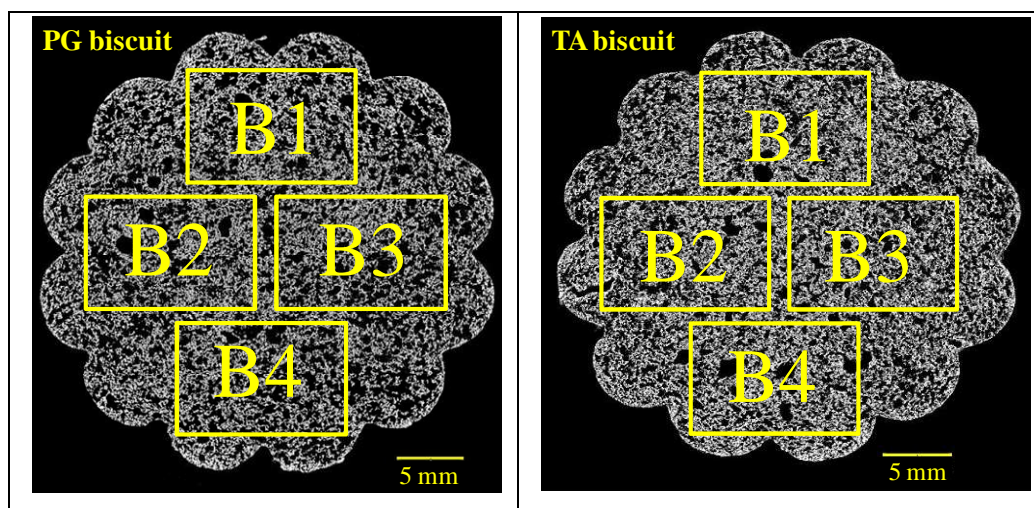


*Figure 6.14 Image from a lateral layer and a vertical slice of PG and TA biscuit with 5 mm scale indicator*

During image analysis, the scale to analyse these images was set up regarding to ‘distance in pixels’ as 1, ‘known distance’ as 0.0225, and ‘pixel aspect ratio’ at 1.0 using mm as ‘unit of length’. Some dark spots shown in the picture during the scan were too small to be accounted as pores, so the noise needed to be removed for the dark outliers at radius of 0.5 pixels. The measurements were then set to analyse the average size of all the pores in the analysed biscuit region and calculated the area fraction (= total pore area ÷ total biscuit area, i.e., porosity). Based on these settings, the results for average pore size and porosity were automatically calculated and recorded using the function of ‘Analyze Particles’ in the ‘Image J’ software.

Initially, all the images from top to bottom layers of the whole biscuit were collected and 100 representative images were selected per biscuit. The middle region (area = 25 x 25 mm) in every biscuit layer was cropped. The settings in the previous paragraph were applied for all six biscuit samples. Total of 300 data for either PG or TA biscuits were generated based on triplicate samples.

Additional investigation was carried out at four different blocks within one biscuit (13.5 x 9 mm) as demonstrated in Figure 6.15. Similar image analysis was carried out for each block per biscuit with 100 image slices. Every biscuit involved 400 images in triplicate samples (either PG or TA biscuits), therefore 1200 slices gathered in total.



*Figure 6.15 Demonstration images of four blocks (B1-B4) selected for both PG and TA biscuit*

### 6.5.2 Aroma Distribution

To investigate vanillin distribution within a biscuit, two vanilla flavourings were freshly prepared (10% w/w vanillin dissolved in PG or TA) and then added at 0.2% into respective dough to be baked at the same tray.

Within one biscuit, seven pieces were cut across with a sharp knife from left to right (A1-A7, as shown in Figure 6.16). Since the size of each piece varied, each piece was weighed and then extracted with methanol and the internal standard (acetovanillone) added for HPLC analysis. Vanillin level in each piece was normalised by its weight. Three PG biscuits and three TA biscuits were used to assess the average vanillin level for each piece.

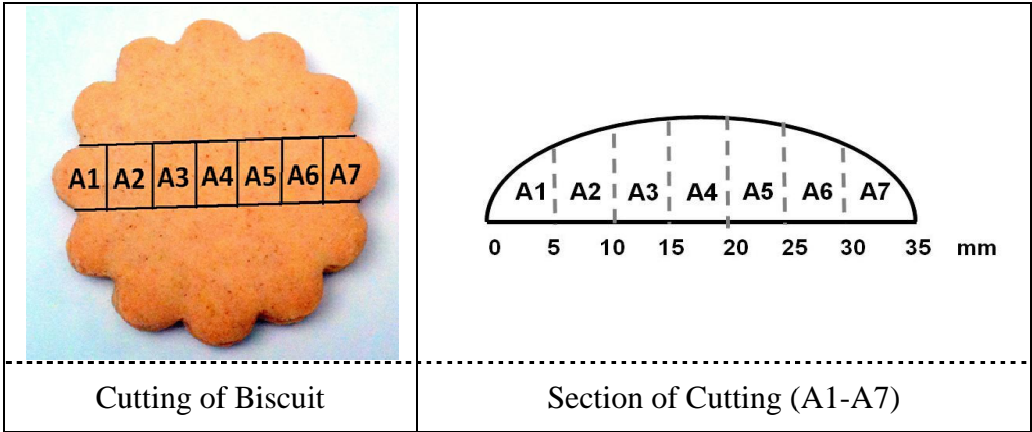


Figure 6.16 Illustration of cutting a biscuit across seven pieces from left to right (A1-A7)

A further three reps of TA biscuits and PG biscuits were cut into four layers from top to bottom (F1-F4, as shown in Figure 6.17). Similarly, each layer was weighed and then extracted by methanol for HPLC analysis and the vanillin concentration was quantified in each layer.

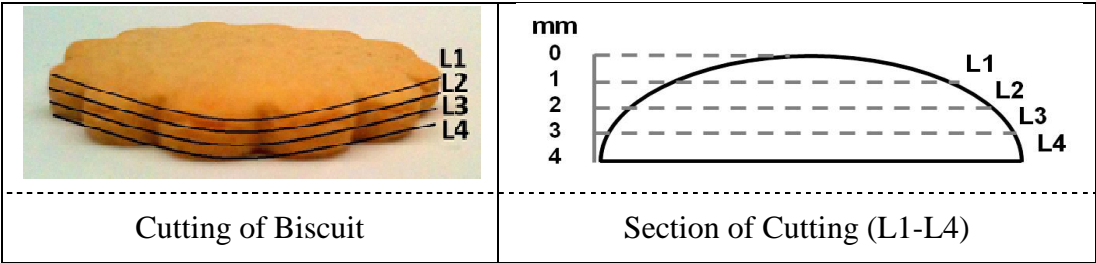


Figure 6.17 Illustration of cutting a biscuit into four layers from top to bottom (L1-L4)

### 6.5.3 Data Analysis for Structure Analysis Results

All results were analysed by SPSS16.0 (SPSS Inc., Chicago, USA) to calculate the average (AV) and standard deviation (STD) of pore size and porosity for both PG and TA biscuits. The histograms fitted with normal distribution curve were created by SPSS Chart Builder. ANOVA-Tukey's MCT was applied to illustrate if there was any significant difference ( $p < 0.05$ ) between two types of biscuits and to identify which selected area was significant different from others (as shown in Figure 6.15). The effect of solvent choice on spatial distribution on vanillin and HMF was evaluated by ANOVA-Tukey's MCT ( $p < 0.05$ ).

Pearson's correlations with two-tailed test were applied to the distribution of vanillin and pores (porosity and average pore size) in the defined areas for both PG and TA biscuit. There is a significant correlation if  $p < 0.05$ .

## **PART III FLAVOUR STABILITY**

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### **7 RESULTS AND DISCUSSION**

The main purpose of this study was to increase understanding to help maintain flavour quality in food products. Specifically, the impact of solvent choice on the stability of flavourings within biscuits during the ASL (accelerated shelf-life) test was chosen for evaluation. As one of the most popular flavourings, a simple vanilla flavouring was used as vanillin dissolved in either PG or TA and applied into shortcake biscuits.

The stability of vanillin in the two solvent systems during biscuit storage was compared and contrasted to the changes of other aroma compounds formed in biscuits, these included HMF generated from non-enzymatic browning reaction during biscuit baking, and 2,4-decadienal and 2,4-heptadienal formed from lipid oxidation during biscuit storage.

The changes in biscuit quality during shelf-life ageing affected not only aroma but also texture; the hardness and fracturability of biscuits during storage was assessed by Texture Analyser for both PG and TA biscuits.

To correlate the aroma and texture changes through instrumental analysis, sensory evaluations were carried out to find out whether these changes could be perceived by consumers. Since the ASL testing uses a higher temperature to shorten the storage period, which may not represent normal storage conditions, additional sensory evaluation of samples stored at ambient temperature with longer periods of time was also conducted. Additionally, the impact of solvent choice on the biscuit micro-structure and aroma distribution within the matrix was studied.



Before conducting the main experiments, it was important to ensure all the biscuits were prepared in a repeatable and reliable way. Since both the colour and the aroma of biscuits are generated through baking, it is vital to control variations from differential heating. Each biscuit at a different location on the tray could be baked to a different extent due to uneven heating from the deck oven. Therefore, the colour, texture and aroma of each individual biscuit in the tray were measured during the preliminary study, and a standardised baking and sampling protocol was defined.

## **7.1 PRELIMINARY STUDY OF BISCUIT BAKING VARIATION**

In addition to analysing biscuit variations due to its locations in the baking tray, the effect of solvent on biscuit colour, texture and aroma was also evaluated.

### **7.1.1 Results of Colour Measurement**

The average results for colour measurement of every biscuit in the baking tray (previously shown Figure 6.5) were calculated from triplicate locations within one biscuit. The results of L, a, b were then plotted (Figure 7.1) in the relevant contour plots with x and y axis as the Location X and Y in the tray respectively.

According to the graphs in Figure 7.1, the x and y axis represent Location X and Y in the tray, while the black dot indicates where a biscuit was located. From the colour measurement of L, a, or b, it was noticed that biscuits at the edge of the tray were at different shading from the rest of the biscuits.

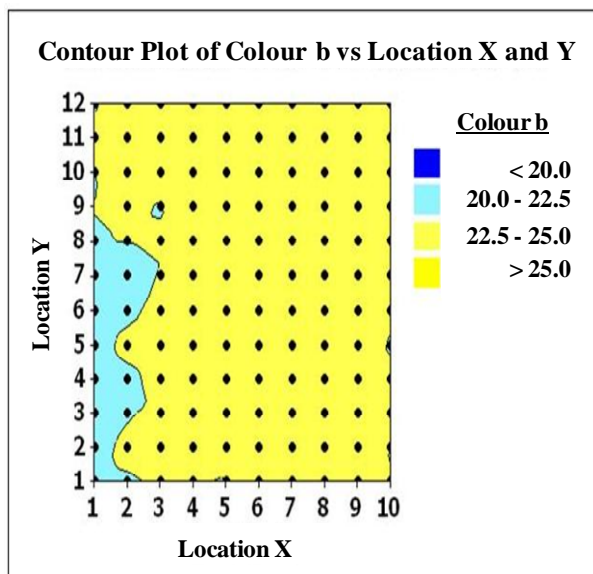
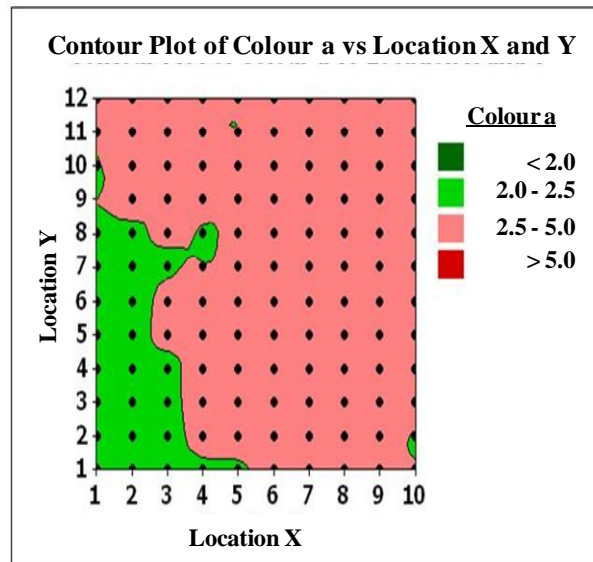
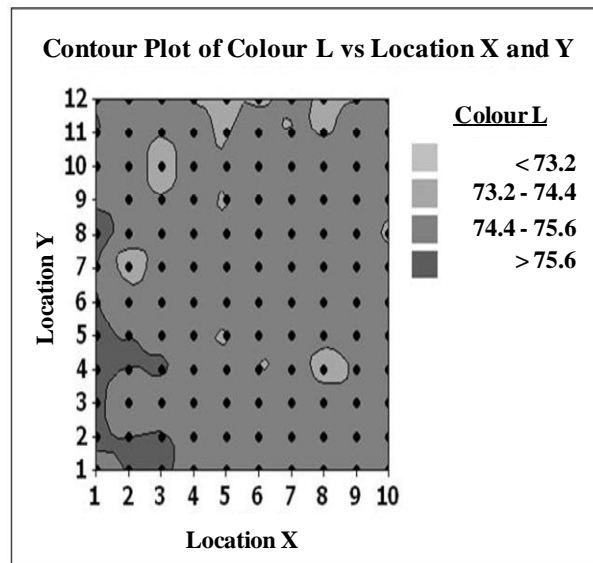
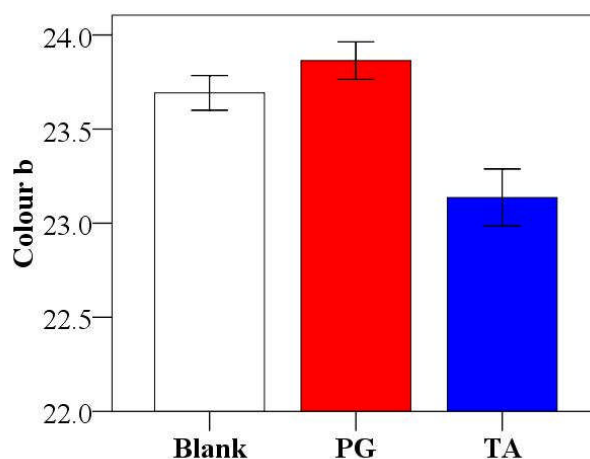


Figure 7.1 Contour plot of colour measurement ( $L$ ,  $a$ ,  $b$ ) and location of the tray ( $X$ ,  $Y$ )

MANOVA indicated that Location X had a significant impact on L ( $p < 0.05$ ), a ( $p < 0.001$ ) and b ( $p < 0.001$ ); while Location Y only had a significant effect on colour b. Regardless of the type of biscuit, the results of colour analysis showed that samples baked at the edge of the tray are significantly different in colour to those in the middle of the tray.

Discarding the variations at the edge of the tray, another MANOVA was applied for the middle samples in the tray ( $2 < X < 10$  and  $1 < Y < 12$ ) when the types of biscuits were taken into account. This result indicated that the use of flavour solvent had no significant effect on the whiteness and redness of the biscuit ( $p > 0.05$ ), but had a significant effect on the yellowness ( $p < 0.001$ ). The average results of colour b (yellowness) for blank, PG and TA biscuit are shown in Figure 7.2. TA biscuits were significantly less yellow than the other two types of biscuits.



*Figure 7.2 Average results of colour b (yellowness) for blank, PG and TA biscuit with error bars representing standard errors*

#### 7.1.2 Results of Texture Measurement

After analysing the colour, each biscuit sample was measured by the Texture Analyser for its hardness (g) - the maximum force to break the biscuit and fracturability (mm) - the distance to the point of break. The less fracturable

biscuits have higher values of fracturability measured. The result of hardness and fracturability measured for every biscuit was plotted with the locations (X and Y) in the tray, as shown in the contour plots (Figure 7.3).

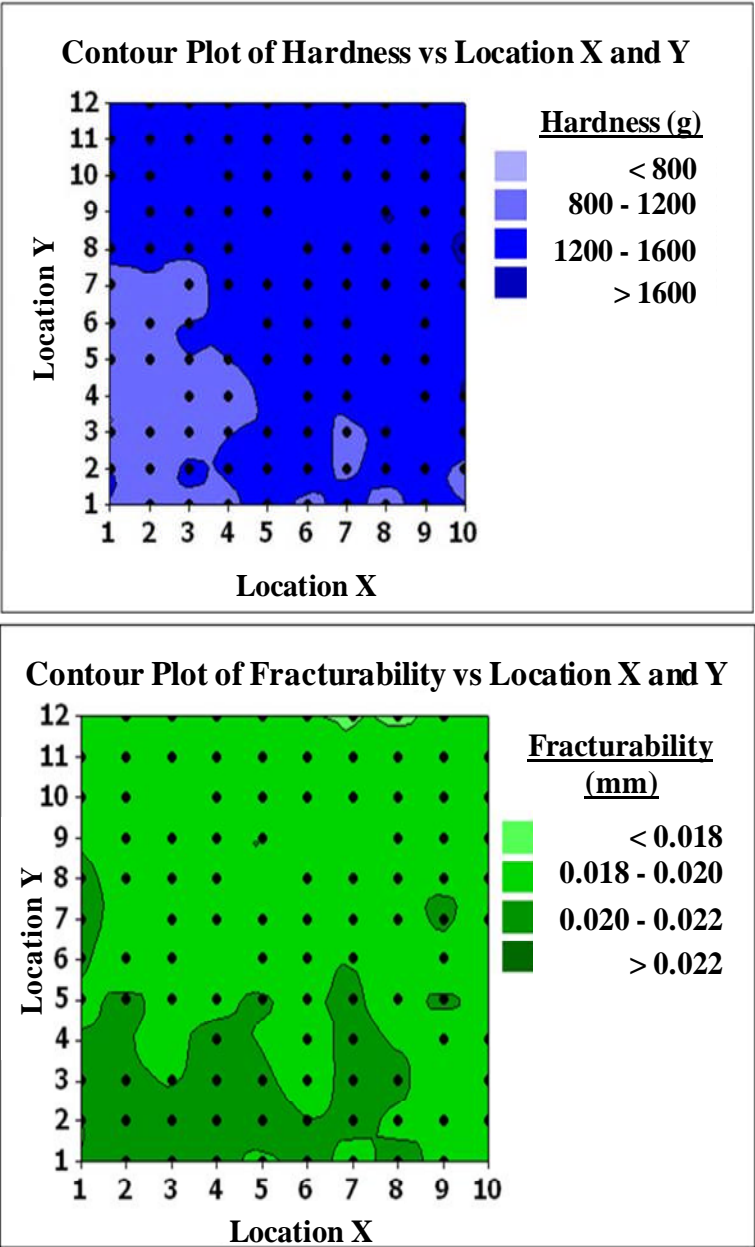
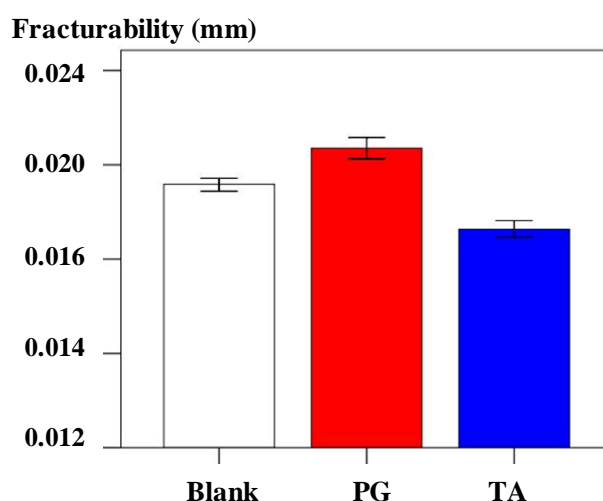


Figure 7.3 Contour plot of texture measurement (hardness and fracturability) and the location of the tray (X, Y)

Generally, the biscuits at the left corner of the tray showed different shading from the rest of the biscuits on the contour plots (Figure 7.3), which indicated they were less hard and less fracturable than other biscuits. MANOVA indicated that the

hardness of the biscuit was significantly affected by Location X ( $p < 0.05$ ) and Y ( $p < 0.05$ ) and that fracturability was significantly affected by Location Y ( $p < 0.001$ ) but insignificant of Location X ( $p > 0.05$ ). Similar to previous colour measurement, the results of the biscuit texture illustrated that the samples baked at the edge of the tray will experience greater variations in baking intensity than those baked at the centre of the tray.

Removing the data obtained from the edge of the tray ( $X = 1, 10, Y = 1, 12$ ), a further MANOVA for all middle biscuits indicated there was no significant solvent impact on biscuit hardness ( $p > 0.05$ ), but a significant impact on its fracturability ( $p < 0.005$ ). As shown in Figure 7.4 for average fracturability results, TA biscuits with smaller values were significantly more brittle than blank and PG biscuits. Therefore, the inclusion of TA as a flavour solvent produced biscuits that were significantly different from the other two systems in terms of colour and texture.



*Figure 7.4 Average results of fracturability (mm) for blank, PG and TA biscuit with error bars representing standard errors*

### 7.1.3 Results of Aroma Measurement

After texture measurement, every biscuit was extracted by methanol added with the internal standard (3-heptanone). Two key biscuit aroma compounds (HMF and furfural) were identified and quantified for every biscuit. The results of the calculated concentration (ppm) for HMF and furfural were plotted with Location X and Y of the tray in the contour plots (Figure 7.5).

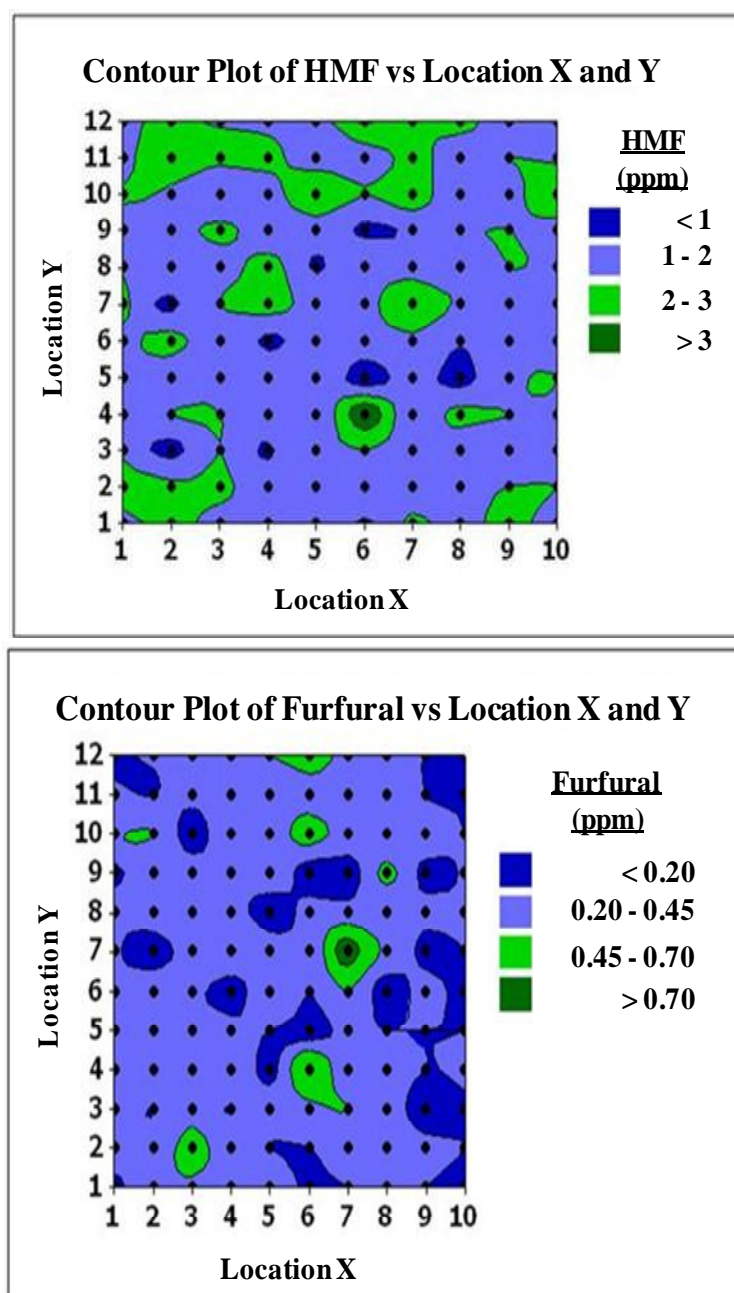
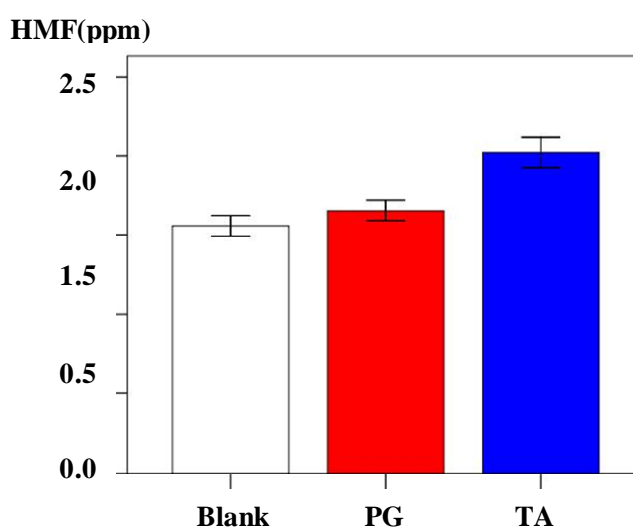


Figure 7.5 Contour plot of aroma measurement (HMF and furfural) and the location of the tray (X, Y)

The colour shade from both contour plots indicated that aroma compounds generated from baking were randomly distributed through the baking tray. There was no significant difference in HMF or furfural concentration between Location X and Y ( $p > 0.05$ ). Compared with previous contour plots from colour measurement, the results indicated that colour generated through Maillard reaction might not be accurately correlated with the amount of HMF or furfural aroma generated. Additional MANOVA for solvent effect indicated no significant impact on the furfural concentration ( $p > 0.05$ ), but a significant impact on HMF concentration ( $p < 0.001$ ) was found. As shown in Figure 7.6, TA biscuits generate significantly higher HMF levels than the levels in blank and PG biscuits.



*Figure 7.6 Average results of HMF (ppm) for blank, PG and TA biscuit with error bars representing standard errors*

To summarise the results of the preliminary study, biscuits baked at the edge of the baking tray were different in colour and texture. As a consequence, only biscuits baked in the middle of the tray were used in remaining studies. Without adding any vanillin, the flavour solvent with 0.2 % addition into the dough showed a significant impact on colour, texture and aroma ( $p < 0.05$ ): TA biscuits were significantly less yellow and more brittle with higher HMF level than PG and blank biscuits.

## 7.2 RESULTS OF INSTRUMENTAL ANALYSIS

The changes of texture and aroma for biscuits stored at ASL test conditions were monitored by instrumental analysis and are reported in Section 7.2.2 and 7.2.1 respectively. The impact of solvent (PG or TA) on texture and aroma stability for all the stored biscuits was evaluated and is shown in Section 7.2.3. The instrumental results of normal storage tests were compared with ASL test results and are detailed in Section 7.2.4.

### 7.2.1 Texture Changes during the ASL Test

After 8 weeks storage, texture analysis was carried out for both PG and TA biscuits previously stored at 20 °C, 32.5 °C and 45 °C with triplicate samples collected weekly at every temperature. As a destructive test, only intact biscuits were used for this test and no replicated measurement could be made.

#### 7.2.1.1 *Changes of hardness*

The results of hardness for biscuit with PG and TA are shown in Figure 7.7 respectively. The starting point was the average hardness result from triplicate control samples for either PG or TA biscuits. The three traces on each graph represent the storage temperatures, and the vertical lines across at every data point are the standard errors calculated from triplicate samples per condition.

As illustrated in Figure 7.7 and confirmed by MANOVA, there was no significant change of hardness observed over the eight week storage time ( $p > 0.05$ ), but biscuits stored at 20 °C were generally less hard than these stored at 32.5 °C and 45 °C ( $p < 0.001$ ).



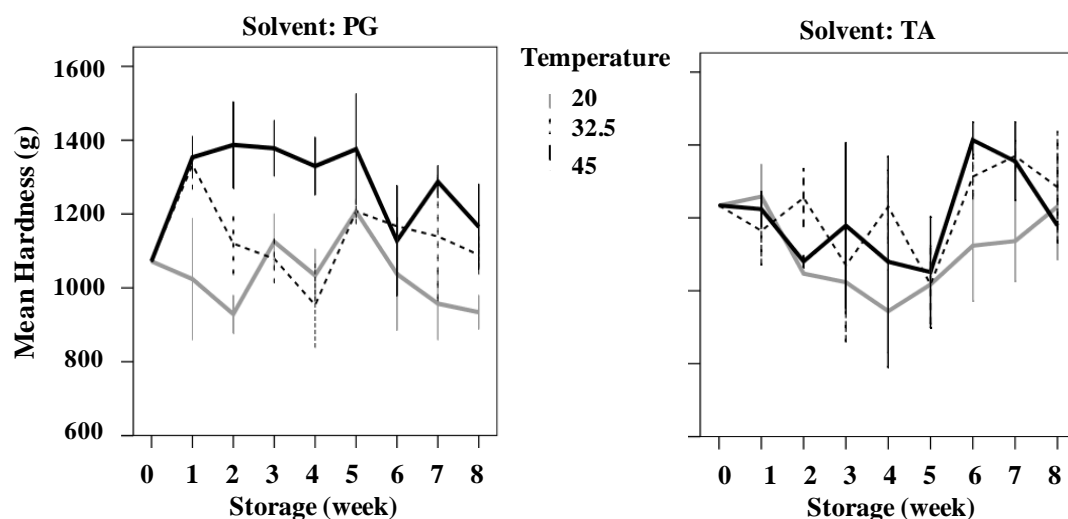


Figure 7.7: Hardness (g) of biscuits prepared with i) PG or ii) TA then stored for 8 weeks at 20 °C (grey-line), 32.5 °C (dotted-line) and 45 °C (black line). Data points are displayed with  $\pm SE$

#### 7.2.1.2 Changes of fracturability

The fracturability for both PG and TA biscuit was measured as the distance to break, and the results are shown in Figure 7.8. MANOVA illustrated no significant fracturability differences during eight weeks storage ( $p > 0.5$ ), but biscuits stored at 20 °C were generally less fracturable than these stored at 32.5 °C and 45 °C ( $p < 0.001$ ).

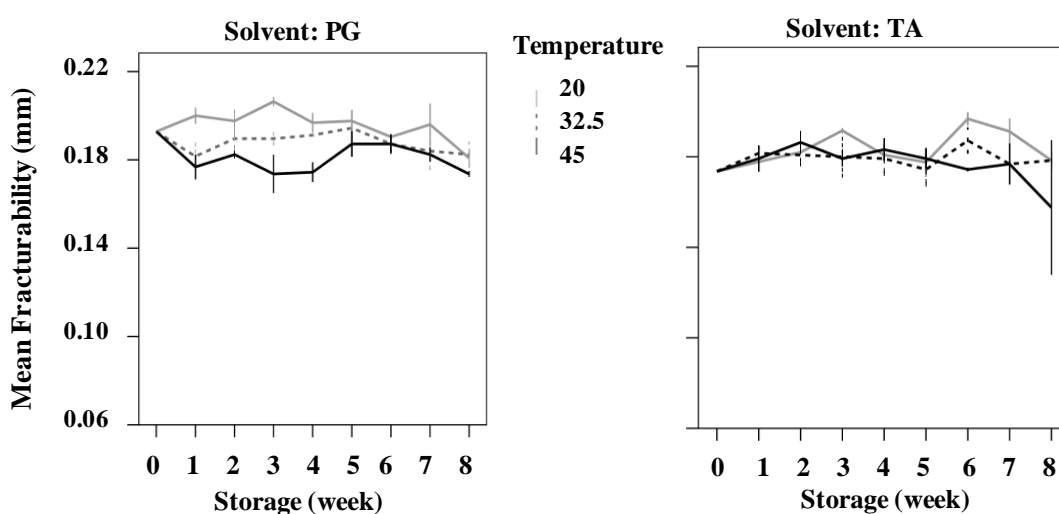


Figure 7.8: Fracturability (mm) of biscuits prepared with i) PG and ii) TA then stored for 8 weeks at 20 °C (grey-line), 32.5 °C (dotted-line) and 45 °C (black line). Data points are displayed  $\pm SE$ .

To summarise the results from texture measurement, neither hardness nor fracturability changed significantly with time of storage up to 8 weeks ( $p > 0.05$ ), but lower storage temperature at 20 °C resulted in less hard and less fracturable biscuits than higher storage temperature at 32.5 °C and 45 °C.

#### 7.2.2 Aroma Changes during the ASL Test

During storage, some aroma compounds might reduce in concentration with time (e.g. volatility, binding, degradation), and some compounds may be generated (e.g. reaction bi-products, such as lipid oxidation secondary products). The major focus of this study was to understand the stability of vanillin in biscuits. No vanillic acid was detected for any of stored biscuits, which might be due to i) the oxidation of vanillin into vanillic acid in the stored biscuit was minimal at the temperature  $\leq 45$  °C; ii) the concentration it formed was below the detection limit.

Although no vanillic acid was detected, some compounds with an oily smell (2,4-decadienal and 2,4-heptadienal) were identified as the marker compounds of lipid oxidation. Additional observations on the changes of biscuit aroma (HMF) that might be an important index for biscuit quality were also reported. The level of furfural in some of the stored biscuits was too low to be detected by GC-MS and HPLC analysis, so its data was not included in this study.

##### 7.2.2.1 *Changes of vanillin level*

The extracted vanillin from biscuits was identified by GC-MS and quantified by HPLC using Equation 6.2. The vanillin concentration ( $\mu\text{g per g biscuit} = \text{ppm}$ ) for every stored biscuit was normalised to its original vanillin level in respective biscuits at week 0, which was regarded as 100%. The use of vanillin% was to avoid different starting point for PG and TA biscuits before storage as these two

solvents might retain different amount of vanillin after baking. The calculated vanillin % for biscuits during storage is shown in Figure 7.9.

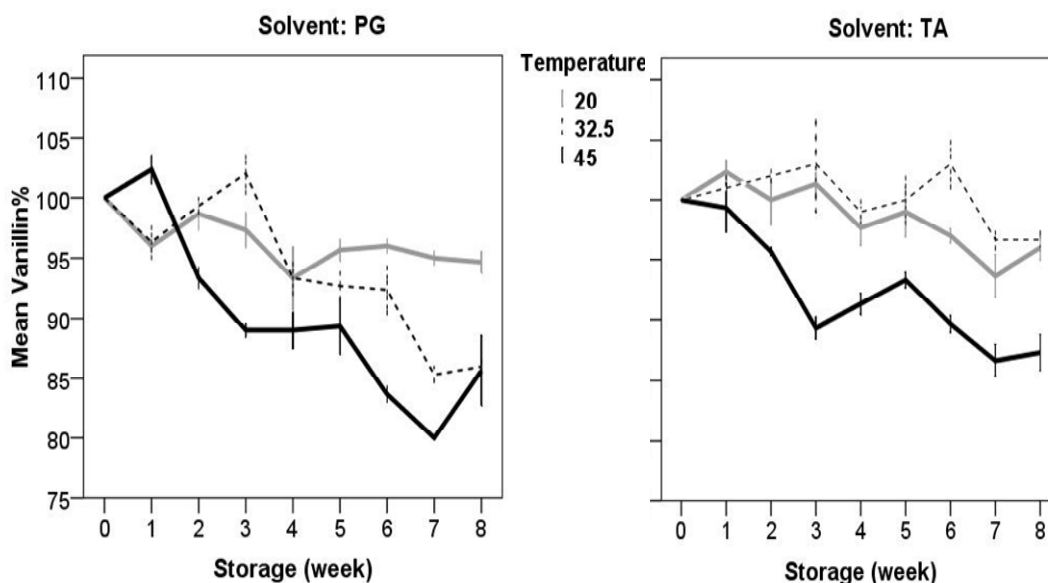


Figure 7.9. Vanillin% in PG and TA biscuits (100% = original concentration of triplicate samples at week 0) analysed weekly after storage for 8 weeks at 20 °C (grey-line), 32.5 °C (dotted-line) and 45 °C (black line). Data points are displayed  $\pm SE$ .

Over the eight week storage test (Figure 7.9), there was a significant ( $p < 0.001$ ) reduction in vanillin concentration in all biscuit samples. This reduction was accelerated at elevated temperatures, i.e., biscuits stored at 45 °C generally showed faster reduction on vanillin level than those at 32.5 °C or 20 °C ( $p < 0.005$ ). The effect of temperature might due to the acceleration of Schiff base formation with increased holding temperature. Nevertheless, up to 80% vanillin was retained in the biscuits after ASL storage. The ability of consumers to identify significant differences of this level is explained in Section 7.3 and the effect of solvent on vanillin stability is evaluated in Section 7.2.3.

#### 7.2.2.2 Changes of HMF level

HMF was identified by GC-MS and quantified by HPLC with concentrations calculated at ppm for all biscuits. Previously Ait Ameer et al. (2006) reported

HMF concentrations in biscuits of 0.5 to 74.6 ppm, within this study HMF concentrations in the fresh biscuits were calculated as 1.7 and 1.9 ppm for PG and TA respectively, which are shown as the starting points in Figure 7.10. Therefore, these levels are within the literature range.

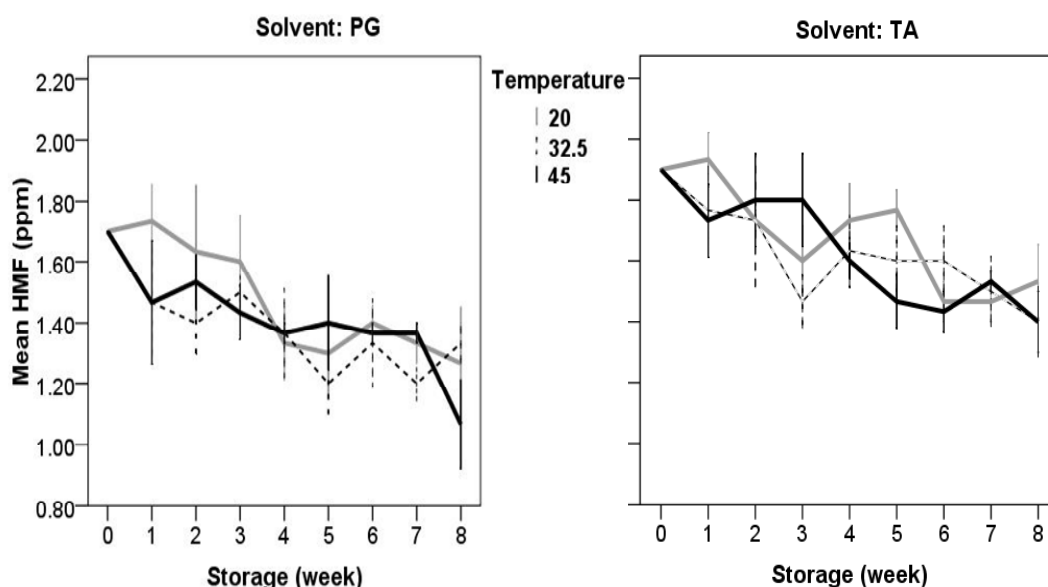


Figure 7.10. HMF concentration ( $\mu\text{g/g}$  biscuit, ppm) in PG and TA biscuits analysed weekly after storage for 8 weeks at 20 °C (grey-line), 32.5 °C (dotted-line) and 45 °C (black line). Data points are displayed  $\pm$  SE

HMF concentration varied with storage time ( $p < 0.05$ ), as detailed in Figure 7.10 for both PG and TA. The progressive loss of HMF over time might be due to a combination of both its inherent volatility and its location of generation: HMF is typically formed at the biscuit surface where the principal browning reactions take place, so it may be sensitive to volatilisation losses during storage (Ait Ameer et al., 2006).

Storage temperature had no significant impact on either PG or TA biscuits ( $p > 0.5$ ), this might be because the maximum storage temperature of 45 °C is not hot enough to further generate HMF (typically produced during roasting/baking reaction chemistries). As a key intermediate of the browning process, the level of

HMF formed is more likely to be affected by the baking temperature. Ameer et al. (2007) reported that cookies baked at 200 °C accumulated 10-100 times less HMF than those baked at higher temperature (250 °C and 300 °C).

#### *7.2.2.3 Changes of lipid off-note level*

During the biscuit storage test, 2,4-decadienal and 2,4-heptadienal were identified and quantified by GC-MS through peak area correction using internal standard (3-heptanone). The concentration of 2,4-decadienal and 2,4-heptadienal for all stored biscuits was calculated as ppm (shown in Figure 7.11 and Figure 7.12 respectively).

For PG and TA biscuits, both 2,4-decadienal and 2,4-heptadienal increased in concentration with time ( $p < 0.001$ ). The storage temperature had a significant impact on their concentrations ( $p < 0.001$ ), i.e., biscuits stored at higher temperature (45 °C) had higher levels of both compounds comparing with those stored at 32.5 °C and 20 °C.

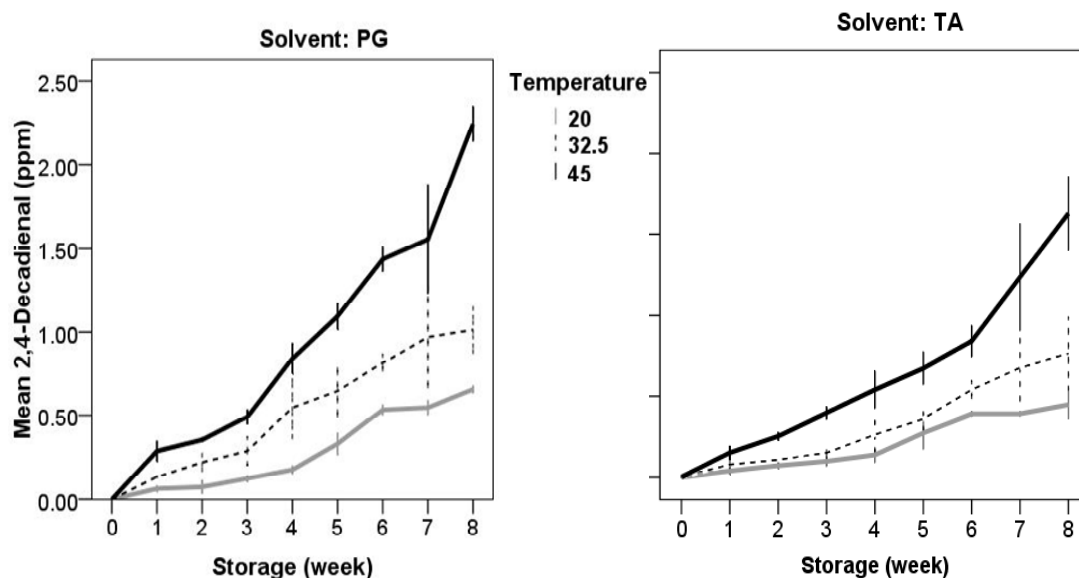


Figure 7.11. 2,4-Decadienal concentration ( $\mu\text{g/g}$  biscuit, ppm) in PG and TA biscuits analysed weekly after storage for 8 weeks at 20 °C (grey-line), 32.5 °C (dotted-line) and 45 °C (black line). Data points are displayed  $\pm$  SE

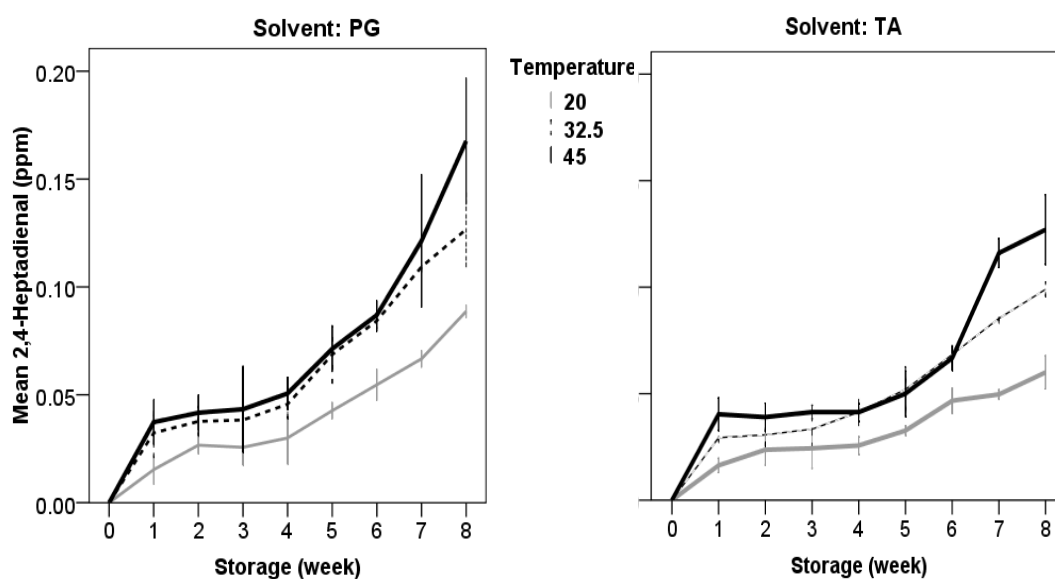


Figure 7.12. 2,4-Heptadienal concentration ( $\mu\text{g/g}$  biscuit, ppm) in PG and TA biscuits analysed weekly after storage for 8 weeks at 20 °C (grey-line), 32.5 °C (dotted-line) and 45 °C (black line). Data points are displayed  $\pm$  SE

### 7.2.3 Impact of Solvent (Changes during the ASL test)

To compare the PG and TA biscuits during storage test, the results of texture and aroma changes were further analysed.

#### 7.2.3.1 Solvent effect on texture changes

Taking all the biscuits stored at the three temperatures into account, the average hardness and fracturability was calculated every week for both PG and TA biscuits, and is plotted in Figure 7.13.

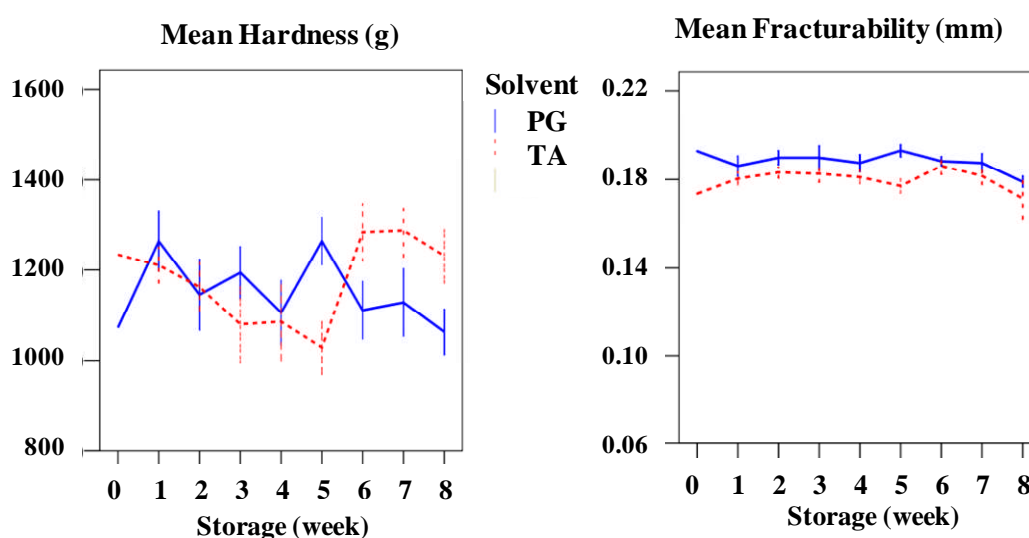


Figure 7.13 Mean hardness (g) and fracturability (mm) calculated from three temperatures during 8 weeks storage for PG biscuits (full line) and TA biscuit (dotted line). Data points are display  $\pm SE$

Comparing pairs of PG and TA biscuits at the same conditions during the eight weeks of storage, ANOVA concludes that there was no significant hardness difference between PG and TA biscuits ( $p > 0.05$ ), but the flavour solvent had a significant impact on the fracturability of the biscuits ( $p < 0.001$ ). Similar to the previous texture measurement results in preliminary studies, TA biscuits with a lower fracturability (mm) were more brittle than PG biscuits.

### 7.2.3.2 Solvent effect on vanillin changes

The stability of vanillin during storage was compared between PG and TA biscuits. The mean normalised vanillin concentration was calculated every week for each storage temperature and is shown in Figure 7.14.

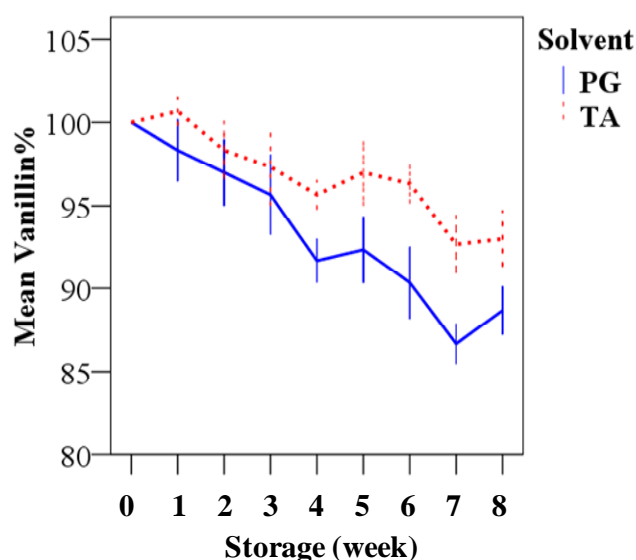


Figure 7.14 Mean vanillin % (100% = concentration at week 0) calculated from three temperatures during 8 weeks storage for PG biscuit (full line) and TA biscuit (dotted line). Data points are display  $\pm$  SE

Biscuits prepared with PG as the flavour solvent exhibited greater losses in vanillin in most cases than those prepared with TA as the carrier solvent (Figure 7.14). One explanation may be the formation of PG-acetal with the aldehyde-vanillin (Elmore et al., 2011). This may be occurring, although it is unlikely as the relative ratios that are normally formed are small and would not account for the significant differences. Another explanation is due to different physicochemical properties between PG and TA, as calculated by EPIsuite<sup>TM</sup>: TA has higher boiling point (260 °C) than PG (188 °C) and TA is less volatile (VP = 0.00248) than PG (VP = 0.129). Those may account for higher retention levels in biscuits after baking (Reineccius, 2006a).



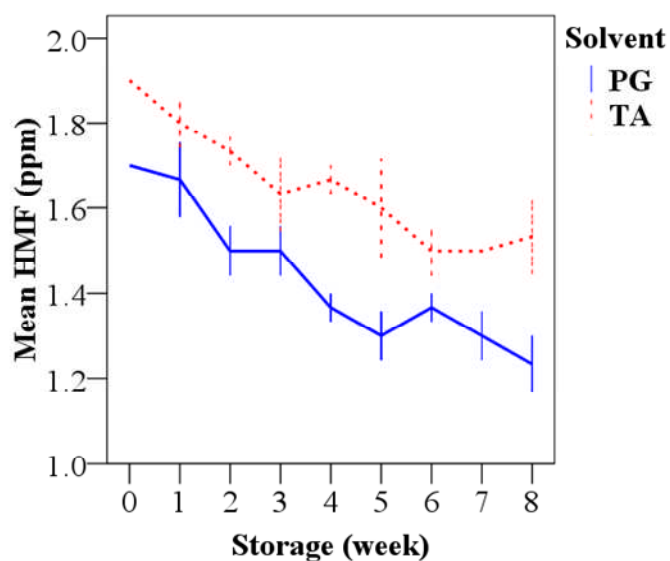
Leclercq et al. (2006) studied the stability of a model aroma mixture during storage, and found that aldehydes were significantly more stable in an oil matrix (medium chain triglyceride) than in water during storage ( $p < 0.05$ ). This may be contributed to the differences in vanillin stability observed in this study. Moreover, TA ( $\text{Log } P = 0.36$ ) is more hydrophobic solvent than PG ( $\text{Log } P = -0.78$ ) and would result in a higher proportion of the flavour residing in the fat. This may account for enhanced stability as the fat phase will confer greater stability during storage as pointed out by de Roos (2006). Therefore, vanillin has greater shelf life stability when dissolved and dispersed in TA than when PG is used as the flavour solvent.

This result may also support the hypothesis that TA as an oily solvent can protect vanillin by retaining it further away from aqueous phase in which the Schiff base condensation can take place. Another hypothesis for the solvent effect on flavour stability may be physical changes in the biscuit structure: i.e. the use of different solvents may generate different biscuit microstructures and thus alter the stability of aroma compounds within the matrix. Further studies to explore the impact of solvent choice on biscuit structure and aroma distribution were carried out and are discussed in Section 7.4.

#### 7.2.3.3 Solvent effect on HMF changes

The impact of solvent choice on the HMF concentration is illustrated in Figure 7.15. TA and PG biscuits had similar pattern of decreasing HMF level with increasing storage time, and TA biscuits had significantly higher HMF level than PG biscuits ( $p < 0.001$ ) after baking. This may be because TA is more hydrophobic than PG, which can lead to a different dough polarity and different

biscuit micro-structure. The rate of loss was similar with both solvents as shown by the two parallel lines.



*Figure 7.15 Mean HMF concentration (ppm) calculated from three temperatures during 8 weeks storage for PG biscuit (full line) and TA biscuit (dotted line). Data points are display  $\pm$  SE*

To sum up, the type of flavour solvent had a significant impact on the stability of the biscuits in terms of fracturability, vanillin and HMF levels plus oily off-notes generated. Compared to PG biscuits, TA biscuits were more brittle with more vanillin and HMF retained in the matrix during storage.

#### 7.2.4 Aroma & Texture Changes during the Normal Storage Test

Comparing the normal stored biscuits (20°C, 24 weeks) with the control samples (-80°C, 24 weeks), no significant impact of time was observed on either hardness or fracturability ( $p > 0.05$ ). Similar to the results of the previous ASL test, solvent had a significant impact on fracturability ( $p < 0.001$ ).

The GC-MS results were analysed by MANOVA, the vanillin level was significantly reduced in the stored biscuits ( $p < 0.001$ ) and the levels of 2,4-decadienal and 2,4-heptadienal were significantly increased ( $p < 0.001$ ). Apart from the significant solvent effect on the vanillin level ( $p < 0.005$ ), no significant solvent impact was observed for the other three aroma compounds ( $p > 0.05$ ). Compared with the ASL results, the level of 2,4-decadienal at normal storage was significantly lower than the level in the ASL test ( $p < 0.05$ ). There was no significant differences for 2,4-heptadienal level between the ASL and normal storage test ( $p > 0.05$ ).

The average vanillin reduction level after ambient storage (20°C, 24 weeks) was calculated as 14% and 9% for PG and TA biscuits respectively. Whilst, the reduced vanillin level after 8 weeks of ASL storage at 45°C was 13% and 10% for PG and TA biscuits. ANOVA compared vanillin results between the ASL and ambient storage, and indicated there was no significant difference on vanillin reduction ( $p > 0.05$ ). The instrumental results indicated that the vanillin reduction level was comparable between the ASL tests using elevated temperatures and a shorter time, when compared to the ambient storage test using a lower temperature for a longer time.

The ambient shelf-life test results in biscuits with less lipid oxidation bi-products than the ASL test, this highlights a limitation of the ASL test and that it might not fully predict all changes in normal conditions. However, the results from the ASL test show a similar vanillin reduction level and this could be used as a rapid evaluation tool to identify some changes during normal storage.

### 7.3 RESULTS OF SENSORY ANALYSIS

#### 7.3.1 Sensory Changes during the ASL Test

Three sessions of paired comparison tests were carried out after 3, 6 and 8 weeks of storage at 45 °C. The stored biscuits were compared with controlled samples (stored at -80 °C) in three pairs with respect to the three attributes that might change during biscuit storage, i.e., brittleness, vanilla flavour and oily off-note. These sensory attributes link to the instrumental measurement of fracturability, vanillin level, 2,4-decadienal and 2,4-heptadienal level respectively. Panellists were asked to choose between controlled and stored samples which biscuit is more brittleness at the first bite, which tasted with stronger vanilla flavour, and which had the strongest oily off-note by smell and taste. The frequency of panellist's selection was recorded for both TA and PG biscuits after 3, 6 and 8 weeks of storage, as summarised in Table 7.1.

*Table 7.1 Number of panellists selected control (CTR, -80 °C) and stored biscuits (Stored, 45 °C) during paired comparison test regarding to brittleness, vanilla flavour and oily off-note for both TA and PG biscuits stored after 3, 6 and 8 weeks*

	Attributes	Samples	3 weeks	6 weeks	8 weeks
TA Biscuits	Brittleness	CTR	14	13	17
		Stored	16	17	13
	Vanilla flavour	CTR	11	16	17
		Stored	19	14	13
	Oily off-note	CTR	15	8	16
		Stored	15	22	14
PG Biscuits	Brittleness	CTR	15	18	14
		Stored	15	12	16
	Vanilla flavour	CTR	13	12	15
		Stored	17	18	15
	Oily off-note	CTR	14	13	10
		Stored	16	17	20

The minimum number of consensual responses required for a total of 30 panellists to conclude that a perceptible difference exists is 21 based on a two-sided paired test (BS-ISO-5495, 2005). According to Table 7.1, only the oily off-note for TA biscuits stored after 6 weeks had responses higher than 22, indicating a significant increase in oiliness after 6 weeks ( $p < 0.05$ ). However, this oily off-note difference in TA biscuits was not present at week 8, which may indicate the complex nature of the aroma profile of a shortcake biscuit is limiting the discriminating ability of the test, and that the concentration difference maybe close to the detection limit of the panellists.

Additionally, the significance level for all paired comparison tests is summarised in Table 7.2. Apart from the significantly oily off-note differences at 6 weeks of TA biscuits, consumers could not discriminate between the biscuits stored at 45 °C after 3, 6 and 8 weeks for brittleness and vanilla attributes ( $p > 0.05$ ). This indicates that the changes observed by instrumental analysis were either sub-threshold or not detectable by the approach taken within the study. On the other hand, with increasing storage time for PG biscuits, the significant level for oily off-notes was inclined to be closer to 0.05, although it did not reach the significance level after 8 weeks.

*Table 7.2 Significant level ( $\alpha$  risk) during paired comparison test in terms of brittleness, vanilla flavour and oily off-note for both TA and PG biscuits stored after 3, 6 and 8 weeks*

<b>Significant (<math>\alpha</math> Risk)</b>		<b>3 weeks</b>	<b>6 weeks</b>	<b>8 weeks</b>
<b>TA biscuit</b>	<b>Brittleness</b>	0.8555	0.5847	0.5847
	<b>Vanilla flavour</b>	0.2005	0.8555	0.5847
	<b>Oily off-note</b>	> 0.9999	<b>0.0161*</b>	0.8555
<b>PG biscuit</b>	<b>Brittleness</b>	> 0.9999	0.3616	0.8555
	<b>Vanilla flavour</b>	0.5847	0.3616	> 0.9999
	<b>Oily off-note</b>	0.8555	0.5847	0.0987

Furthermore, there were limitations of these sensory tests due to the changeover in panel composition involved in three discontinuous sessions and the learning effect for some panellists. As a result, generating more consistent panel responses over time might be difficult for these tests.

### 7.3.2 Sensory Changes during the Normal Storage Test

During sensory analysis for normal stored biscuits, similar attributes as previously selected (the brittleness, vanilla flavour and oily off-note) were assessed for both PG and TA biscuits by 30 panellists. The sensory results including the number of right answers and significant level for every pair were summarised in Table 7.3.

*Table 7.3 Number of right answers and significant level ( $\alpha$  risk) in the paired comparison tests comparing control samples (CTR, -80 °C) with stored biscuits (Stored, 20 °C) after 24 weeks in terms of brittleness, vanilla flavour and oily off-note for both TA and PG biscuits*

	Attributes	Samples	Right Answers	Significant ( $\alpha$ Risk)
<b>TA Biscuits</b>	<b>Brittleness</b>	CTR	13	0.5847
		Stored	17	
	<b>Vanilla</b>	CTR	16	0.8555
		Stored	14	
	<b>Oily off-note</b>	CTR	15	1.0000
		Stored	15	
<b>PG Biscuits</b>	<b>Brittleness</b>	CTR	14	0.8555
		Stored	16	
	<b>Vanilla</b>	CTR	<b>21</b>	<b>0.0428*</b>
		Stored	<b>9</b>	
	<b>Oily off-note</b>	CTR	18	0.3616
		Stored	12	

A significant difference ( $p < 0.05$ ) was perceived for PG biscuits when 21 out of 30 panellists chose the controlled sample having a stronger vanilla flavour than the stored sample. It indicated that the reduced vanillin level in these PG biscuits stored at ambient temperature for a longer period of time up to 24 weeks was

significantly perceivable. In addition, consumers could not discriminate the brittleness between the control and stored biscuits and there was no significantly perceivable oily off-note after storage ( $p > 0.05$ ).

The significance levels can be compared with previous results from the ASL test (Table 7.2). The comparison between significant levels at normal conditions (20 °C, 24 weeks) and ASL conditions (45 °C, 8 weeks) for both PG and TA biscuits was summarised in Figure 7.16. When the value falls below the dashed line at 0.05, there is a significant difference observed after storage. The higher the bar, the less likely there is a significant difference.

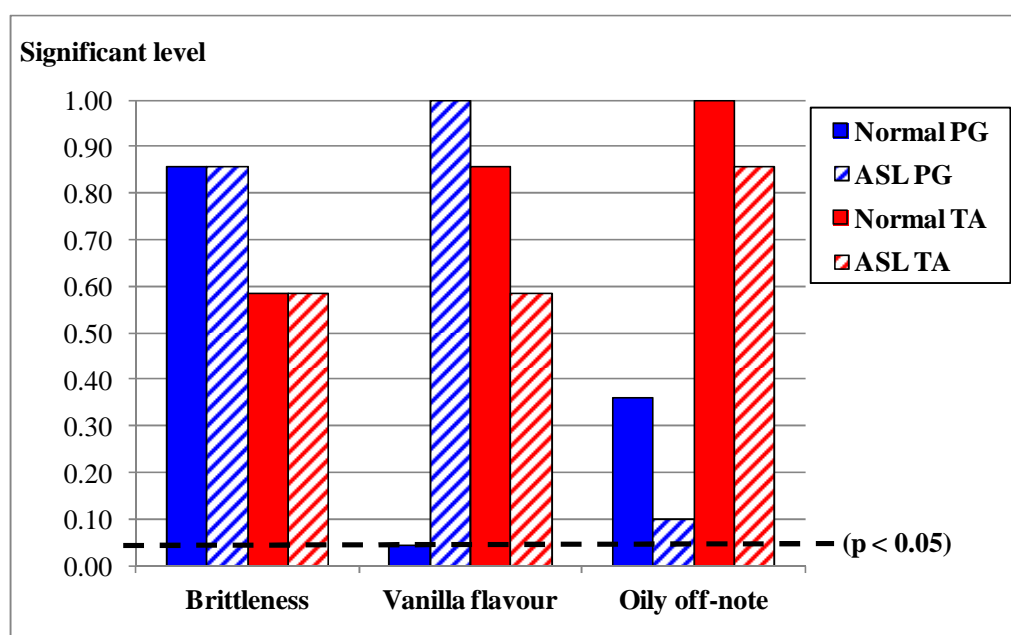


Figure 7.16 Summary of significant level obtained from paired comparison test at normal storage test (20 °C, 24 weeks) for PG biscuits (shown in full blue bar) and TA biscuits (shown in lined blue bar), comparing with ASL test (45 °C, 8 weeks) for PG biscuits (shown in full red bar) and TA biscuits (shown in lined red bar) in terms of brittleness, vanilla flavour and oily off-note.

Despite PG or TA biscuits, the significant level on brittleness was the same for both normal storage and ASL test, so neither types of test have a significant impact on the brittleness of the biscuits ( $p > 0.05$ ). The oily off-note for all the biscuits was not significant ( $p > 0.05$ ), but the significance level for either PG or



TA biscuits at the ASL test was lower than its respective level in normal conditions, so the ASL test with a higher storage temperature might be more likely to induce relevant changes in the oily off-note. In general, PG biscuits with lower bars than TA biscuits are more likely have a significant oily off-note.

Regarding the vanilla flavour, normally stored PG biscuits showed a significant difference ( $p < 0.05$ ) but the ASL stored PG biscuits showed no significant difference. Although no significant changes were perceived for all TA biscuits, the ASL storage might be more likely to have a potential impact on vanilla flavour perception.

To correlate the above sensory results with previous instrumental findings, the oily off-note (2,4-decadienal) was significantly higher in the ASL test (45 °C, 8 weeks) than in normal storage ( $p < 0.05$ ), so it is possible that the oily off-note can be perceived more in biscuits stored at ASL test. On the other hand, the measured vanillin reduction level was not significant between the ASL test and normal storage for PG biscuits (13% v.s. 14%) and TA biscuits (10% v.s. 9%), but the sensory results indicated that panellists could only differentiate the vanilla flavour intensity for the normal stored biscuits with up to 14% reduction than in the controlled biscuits. Therefore, it is important to confirm the detectable difference level for vanillin in biscuits, as shown in the follow section.

### 7.3.3 Detectable Difference Test for Vanillin in Fresh Biscuits

Additional sensory tests were carried out for biscuits with the original vanillin level and a reduced vanillin level, triangle tests were used to evaluate the impact of biscuits made at three different vanillin levels (V1= 10% reduction, V2= 20% reduction and V3= 30% reduction).

The actual vanillin levels in those biscuits from GC-MS analysis were calculated and the reduction percentage for biscuits with V1, V2 and V3 flavouring was compared to the biscuit with original V0 flavouring. The calculated results were 10.6%, 21.4% and 30.9% for V1, V2 and V3 biscuits respectively. These three values were close to the designed reduction levels, so they were accepted for use in sensory evaluation.

With thirty normal biscuit consumers involved in the triangle test, the minimum number of correct responses required is 15 to conclude a significant difference at  $\alpha$  risk of 0.05 (BS-ISO-4120, 2004). As shown in Table 7.4, no significant sensorial difference was observed between the biscuits with original level and with vanillin up to 30% reduction level. This might due to the fact that biscuit is a sweet matrix and majority of consumers are not be able to differentiate the added sweet vanilla flavour from the sweet biscuit matrix or that simply the difference is not perceivable. Future work could be conducted for biscuits with no vanillin and added vanillin in order to check if consumers could distinguish vanilla flavour in biscuits and at what level. Vanillin content could be further reduced by as much as 50% compared to its original level to explore the just noticeable difference level in biscuits.

*Table 7.4 Number of right answers and significant level ( $\alpha$  risk) for three triangle tests including fresh biscuit with vanillin at a) original level and 10% reduction; b) original level and 20% reduction; c) original level and 30% reduction*

Test	Right Answers	Significant ( $\alpha$ Risk)
a). Original/ 10% Reduction	13	0.1660
b). Original/ 20% Reduction	11	0.4152
c). Original/ 30% Reduction	11	0.4152

The results of the vanillin detectable difference test for these fresh biscuits were compared directly with the previous sensory tests for the stored biscuits. The

vanillin level was reduced up to 10% reduction in TA biscuits during 8 weeks storage at 45°C, at which no significant difference in vanilla flavour was perceived. This is in agreement with the results of the fresh biscuits that consumers could not detect the difference at 10% vanillin reduction.

However, the normal stored TA biscuits (24 weeks 20 °C) with 9% vanillin reduction were perceived to be significantly different, which indicated a contradictory finding from this test. A possible reason might be due to random variation and the type of question asked in the sensory test. In the paired-comparison test for normal stored biscuits, the discrimination based on vanilla flavour intensity was pointed out and therefore was the focus of the assessor's attention; whilst in the triangle test, panellists were asked to determine the overall difference. Therefore greater background noise is created and less attribute specific focus which will increase the difficulty of effective discrimination.

To confirm the results of a no detectable difference for biscuits with 30% vanillin reduction, a paired comparison test for biscuits at the original and reduced vanillin levels could be repeated in the future. Furthermore, more panellists (up to 90) are required to conclude 30% vanillin reduction had a similar vanilla flavour as the original level in biscuits (BS-ISO-5495, 2005).

To conclude, the results indicate that up to 20% of the vanillin in biscuits could be removed with no significant difference as perceived by many consumers; the subsequent reduction in vanillin could be significant, although this may alienate some consumers.

## 7.4 RESULTS OF STRUCTURE ANALYSIS

### 7.4.1 Results of X-ray Analysis

Based on the image analysis results obtained from an X-ray CT scan, the histogram (Figure 7.17) shows the average pore size distribution for both PG and TA biscuits with the average pore size (AV) and the standard deviation (STD) calculated from a total (N) of 300 data points.

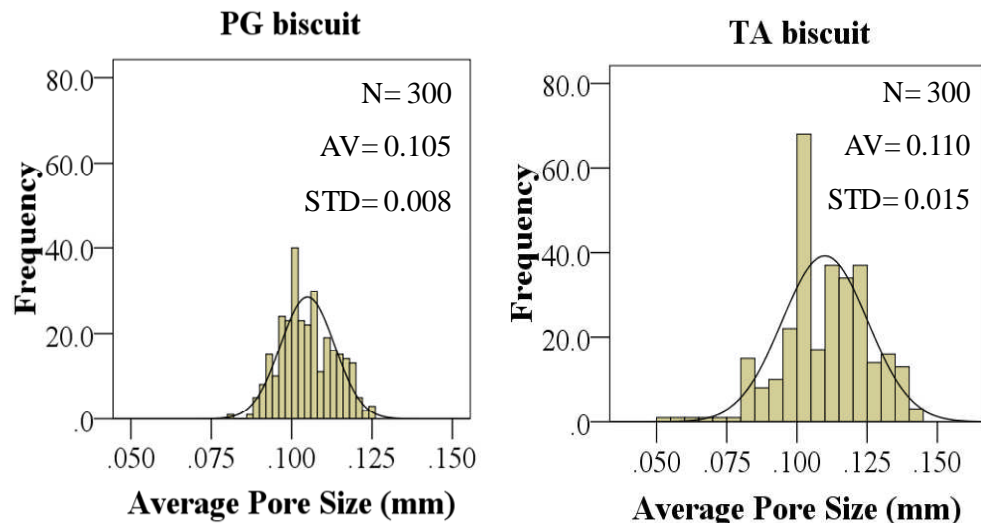
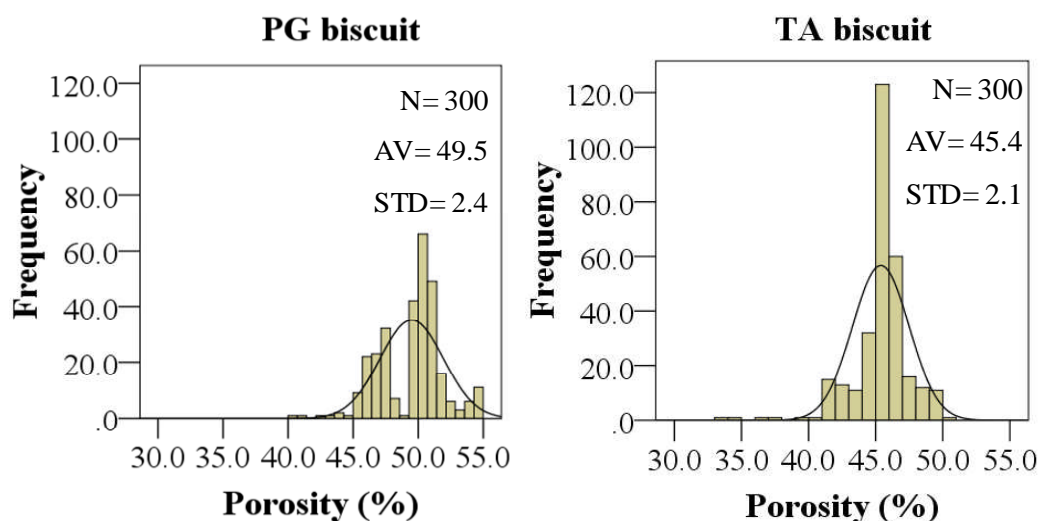


Figure 7.17 The histograms illustrated the distribution of average pore size (mm) for PG and TA biscuit with fitted normal curve. The average pore size (AV) and standard deviation (STD) were calculated from total of 300 data.

In Figure 7.17, the distribution for both PG and TA biscuits showed higher frequency at the average pore size around 0.100 mm. The average pore size for PG biscuits ( $0.105 \pm 0.008$  mm) was significantly larger than the size of the TA biscuits ( $0.110 \pm 0.015$  mm) at  $p < 0.001$  by ANOVA.

Similarly, the area fraction (porosity) for PG and TA biscuits was analysed and is shown in the histograms Figure 7.18, with respective average porosity (AV) and standard deviation (STD) from 300 data points.



*Figure 7.18 The histograms illustrated the distribution of porosity (%) for PG and TA biscuit with fitted normal curve. The average porosity (AV) and standard deviation (STD) were calculated from total of 300 data.*

Although the histogram of porosity (%) for both PG and TA biscuits was not strictly a normal distribution (Figure 7.18), ANOVA of the average porosity (%) of PG biscuits ( $49.5 \pm 2.4$  %) and TA biscuits ( $45.4 \pm 2.1$ %) indicated a significant difference ( $p < 0.001$ ). This indicated that PG biscuits were significantly more porous than TA biscuits

In a short summary, the results of the X-ray scan indicated that PG biscuits have more pores at smaller size while TA biscuits have less pores but at larger pore size. To further understand the difference 4 internal blocks within the biscuit (illustrated previously in Figure 6.15) were analysed for pore size. The calculated average pore size at each block for PG and TA biscuits was shown in Figure 7.19.

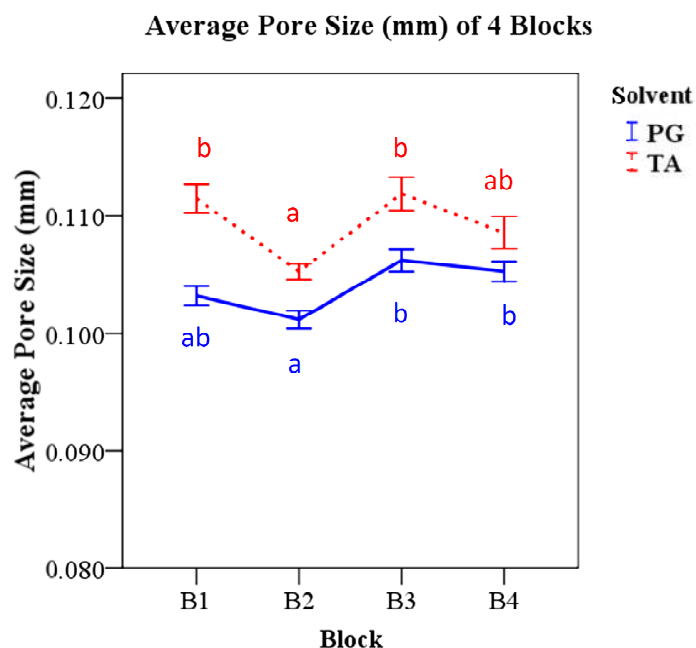


Figure 7.19 Results of average pore size (mm) calculated for 4 blocks (B1-B4) within PG biscuits (full line) and TA biscuits (dotted line). Data points are displayed with  $\pm$  SE, different letters indicated significant different at  $p < 0.05$ .

As shown in Figure 7.19, the average pore size for all TA blocks was larger than the PG biscuits, a significant effect of solvent type ( $p < 0.001$ ) and locations of the block ( $p < 0.001$ ) was shown by MANOVA. Despite the choice of solvent (PG and TA), block B2 had significantly smaller pores than other blocks ( $p < 0.05$ ) as detailed by Tukey's MCT, although this might be a random difference.

Similarly, the average porosity at each block was calculated for both solvent types and is shown in Figure 7.20. In general, PG had a significant larger porosity than TA ( $p < 0.001$ ). The porosity at block B1 was significantly higher than the other three blocks for TA biscuits ( $p < 0.05$ ), but no significance between the four blocks of PG biscuits ( $p > 0.05$ ) was identified.

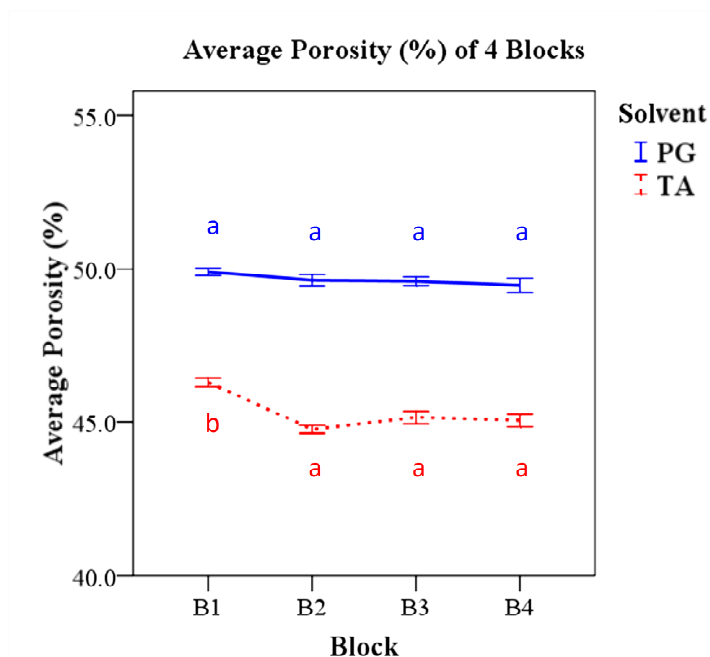


Figure 7.20 Results of average porosity (%) calculated for 4 blocks (B1-B4) within PG biscuits (full line) and TA biscuits (dotted line). Data points are displayed with  $\pm$  SE, different letters indicated significant different at  $p < 0.05$ .

Overall, the key findings from the four blocks analysis were that TA biscuits were confirmed to be less porous with larger pores compared to PG biscuits.

#### 7.4.2 Results of Aroma Distribution Analysis

##### 7.4.2.1 Cutting across seven pieces

###### (A) Vanillin level across 7 pieces

Biscuits of both solvent type were formulated by the standard preparation protocol and then a single cross sectional slice was taken which was subsequently sectioned into seven cross sectional pieces. The vanillin concentration was calculated in ppm from all seven pieces for three PG biscuits (P1, P2, P3) and three TA biscuits (T1, T2, T3) were shown in Figure 7.21.

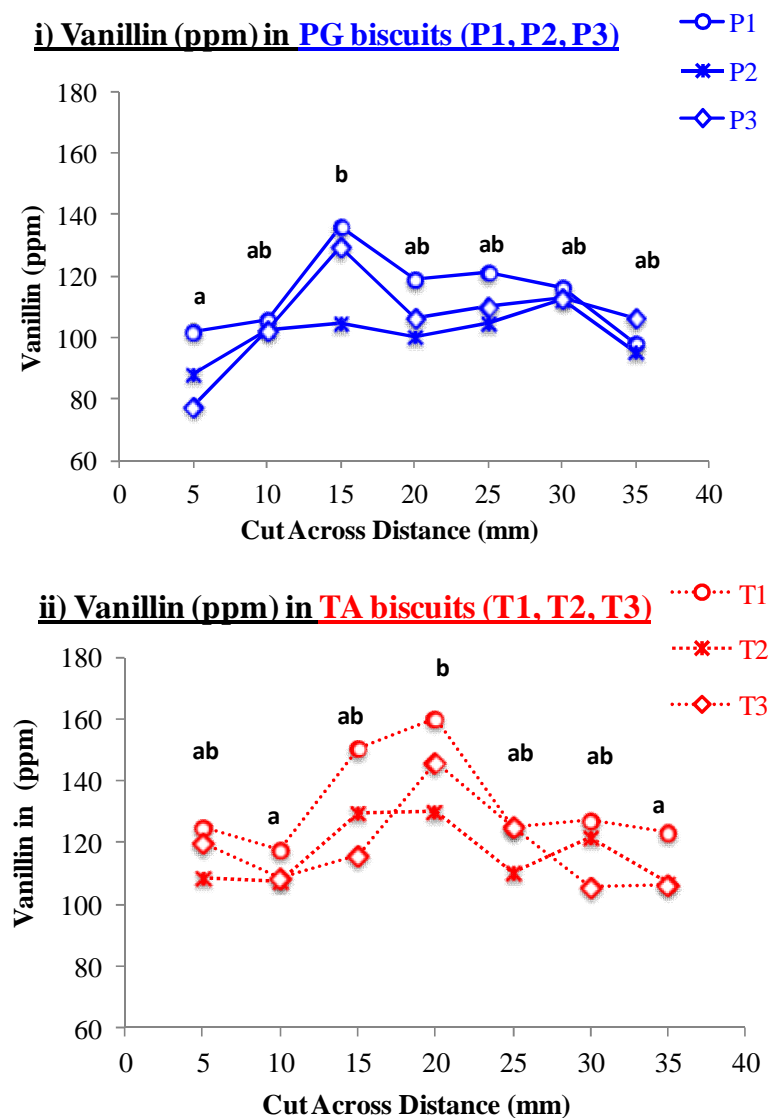


Figure 7.21 Distribution of vanillin concentration (ppm) cutting across seven pieces for i) three PG biscuits (P1, P2, P3) and ii) three TA biscuits (T1, T2, T3). Different letters indicated significant different levels between pieces ( $p < 0.05$ ).

Similar patterns of vanillin distribution across the biscuit were observed in Figure 7.21, i.e., significantly higher vanillin content in the middle of the biscuit (15-20 mm) than at the edge ( $p < 0.05$ ). This might be due to the Maillard reaction occurring at a higher rate at the edge of the biscuit, or that heating it might induce more vanillin to be evaporated at the edge. When comparing the average vanillin concentration at each location, there is a significant difference between PG and TA biscuits ( $p < 0.05$ ) based on ANOVA, which indicated a significantly higher vanillin concentration observed in TA biscuits.



**(B) HMF level across 7 pieces**

Additionally, the concentration of HMF was calculated for three biscuits of PG and TA, as shown in Figure 7.22.

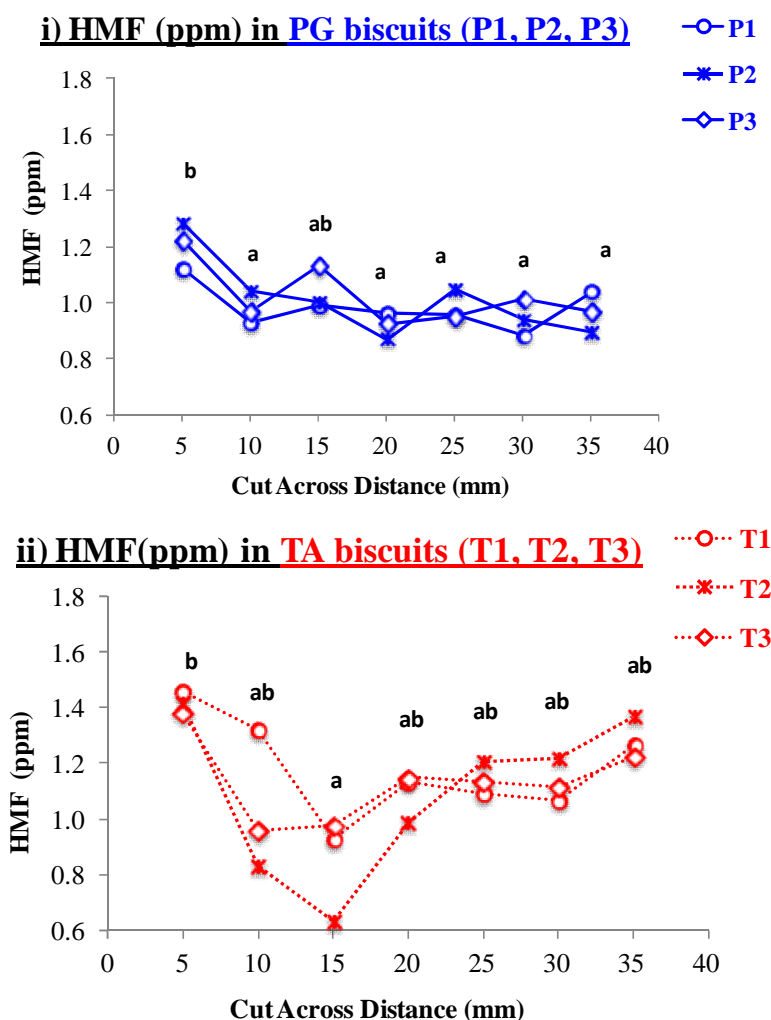


Figure 7.22 Distribution of HMF concentration (ppm) cutting across seven pieces for i) three PG biscuits (P1, P2, P3) and ii) three TA biscuits (T1, T2, T3). Different letters indicated significant different levels between pieces ( $p < 0.05$ ).

Both PG and TA biscuits showed a significant location difference on the HMF concentration ( $p < 0.005$ ). The first piece in all three PG biscuits had a significantly higher HMF concentration than in the other pieces ( $p < 0.05$ ), which might be caused by uneven Maillard reaction across the biscuits or may also be due to spatial differences in the dehydration rate. As the biscuits can be turned

around, this is more likely to be a random difference. ANOVA indicated no significant HMF concentration difference between PG and TA biscuits ( $p > 0.05$ ).

#### 7.4.2.2 Cutting into four layers

##### (A) Vanillin level from 4 layers

Triplicate biscuit samples (PG or TA) were cut from top to bottom into four layers, and the average vanillin concentration of all three samples for each layer is shown in Figure 7.23.

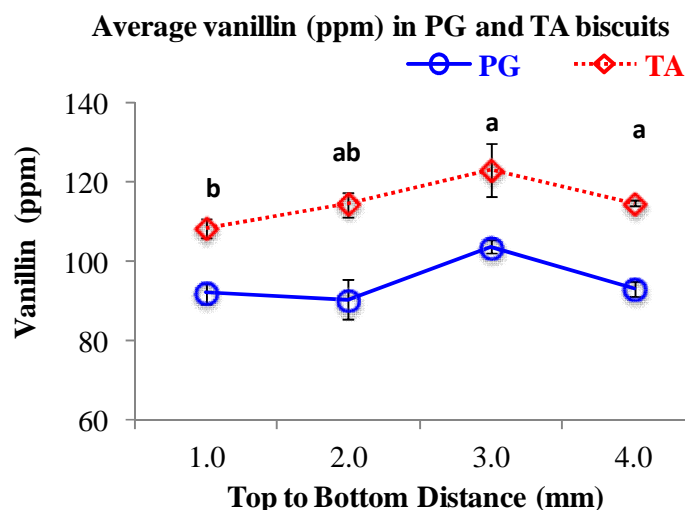
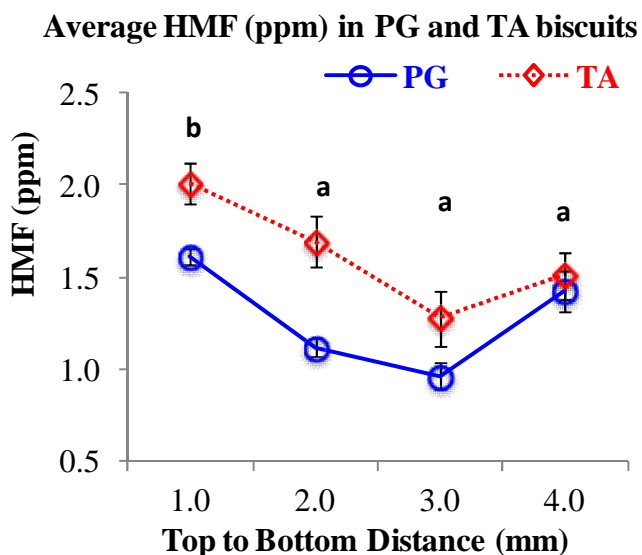


Figure 7.23 Average vanillin concentration (ppm) calculated for four layers cutting from top to bottom for PG and TA biscuits with error bars displayed as  $\pm$  SE. Different letters indicated significant different levels between layers ( $p < 0.05$ ).

Both PG and TA showed a similar pattern of vanillin distribution from the top to the bottom of the biscuit: a significantly higher vanillin concentration ( $p < 0.05$ ) in the bottom layer (4 mm) than in the top layer (1 mm). The effect of the solvent was significant ( $p < 0.005$ ): TA biscuits had significantly higher vanillin concentrations than PG biscuits, as agreed with all previous findings.

***(B) HMF level from 4 layers***

The average concentration of HMF (ppm) for both PG and TA biscuits is shown in Figure 7.24, the surface at the top of the biscuit (1 mm) of both PG and TA biscuits had a significantly higher HMF concentration ( $p < 0.05$ ). This is because due to higher rates of the Maillard reaction occurring at the biscuit surface (Capuano & Fogliano, 2011). There was a significant impact of solvent choice on HMF concentration ( $p < 0.05$ ).



*Figure 7.24 Average HMF concentration (ppm) calculated for four layers cutting from top to bottom in PG and TA biscuits. Error bars displayed as  $\pm$  SE, different letters indicated significant different levels between layers ( $p < 0.05$ ).*

To summarise the results from the aroma distribution study, vanillin concentrations remained significantly higher in the centre of the biscuit matrix than at the edge or surface for both PG and TA biscuits, this might be due to vanillin loss through evaporation from the matrix into the air, additionally, the concentration of the baked aroma compound originating from the Maillard chemistries, HMF, was higher at the biscuit surface.

### 7.4.3 Correlation between Vanillin Distribution and X-ray Results

In order to investigate the relationship between the vanillin distributions and the biscuit structure that were both affected by the type of flavour solvent added, additional image analysis was applied to both PG and TA biscuits. The mathematical cropping was comparable to the physical cross sectioning as detailed previously, seven regions (4 x 4 mm) were cropped from the images and the relevant structure parameters for each region was calculated based on 100 cross sectional image slices for both solvent types (PG or TA), again this was conducted in triplicate samples. Of the 4200 generated data points, the average pore size (mm) and porosity (%) from the seven pieces are shown in Figure 7.25.

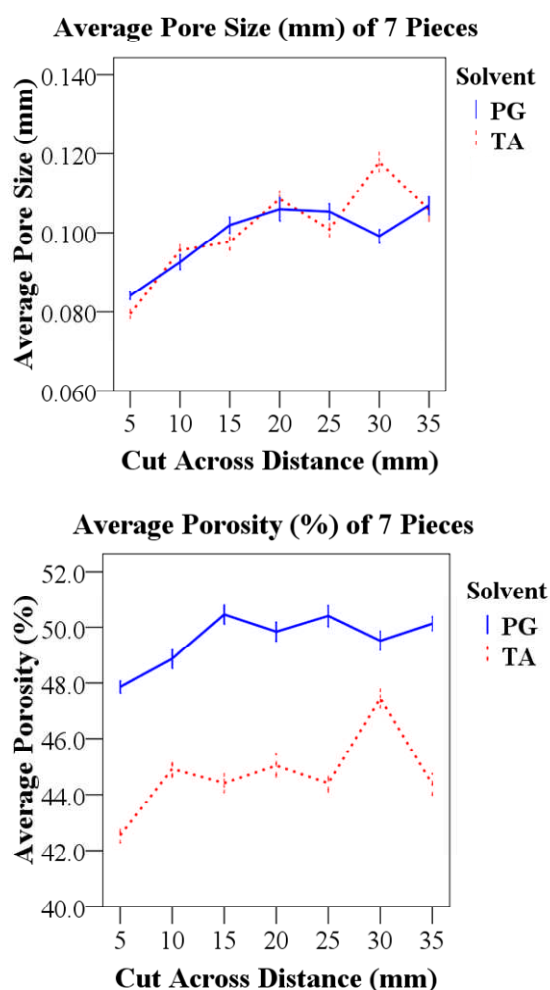


Figure 7.25 Average pore size (mm) and porosity (%) calculated for seven pieces cutting across PG and TA biscuits. Error bars displayed as  $\pm SE$

Further data analysis from Figure 7.25 indicated that both average pore size (mm) and average porosity (%) was dependent on the location of the pieces significantly for both PG and TA biscuits ( $p < 0.001$ ). PG biscuits were significantly more porous than TA biscuits ( $p < 0.001$ ), but the average pore size between PG and TA biscuits was not significantly different ( $p > 0.05$ ). The lack of statistical difference on average pore size can be explained by the reduced areas of analysis in every piece ( $16 \text{ mm}^2$ ), although an indication of the difference between PG and TA biscuits can be seen previously at  $121.5 \text{ mm}^2$  cross four internal blocks (see Figure 7.19).

Compared with the distribution of vanillin, the edge of the biscuits was significantly less porous ( $p < 0.001$ ) and had significantly retained less vanillin ( $p < 0.05$ ). Pearson's correlations with the two-tailed test indicated a significant correlation ( $p < 0.05$ ) between average vanillin concentration and average porosity for PG biscuit ( $r = 0.768$ ), but not significant for TA biscuit ( $p > 0.05$ ). Average pore size was not significantly correlated to the vanillin concentration in either PG or TA biscuits ( $p > 0.05$ ).

Further image analysis was applied to four layers with 40 image slices at each layer from top to bottom of the biscuit. The results of the average pore size (mm) and porosity (%) from these four layers are shown in Figure 7.26. Two middle layers (2.0 - 3.0 mm) had significantly larger pores ( $p < 0.001$ ) and larger porosity ( $p < 0.001$ ) than both edges of the biscuits. The choice of flavour solvent significantly impacted average pore size ( $p < 0.001$ ) and porosity ( $p < 0.001$ ) with TA biscuits having larger pores but lower porosity than PG biscuits, this confirms previous findings (Section 7.4.1).

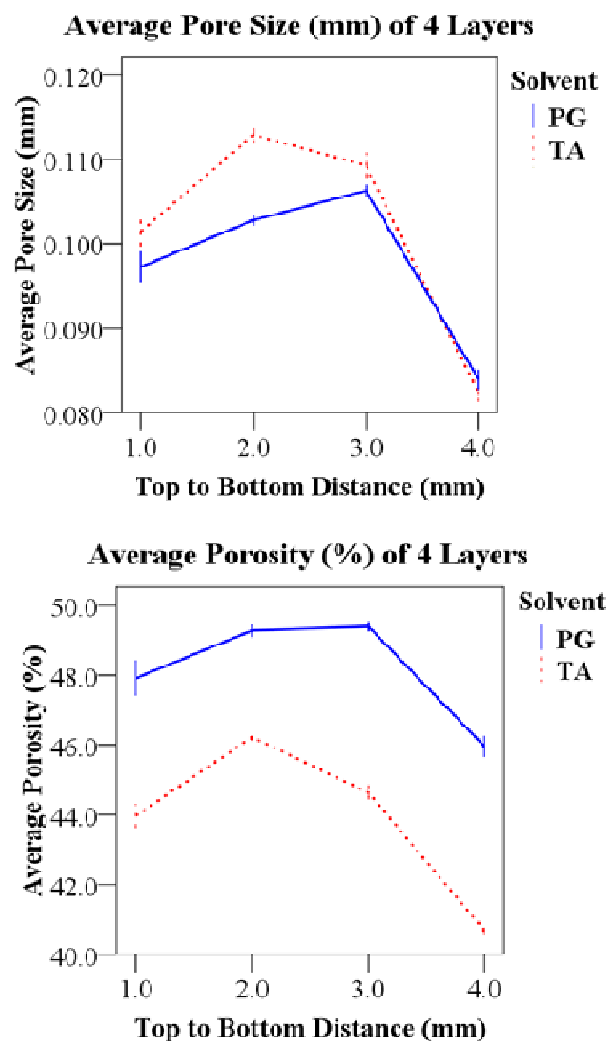


Figure 7.26 Average pore size (mm) and porosity (%) calculated for four layers cutting from top to bottom of PG and TA biscuits. Error bars displayed as  $\pm SE$

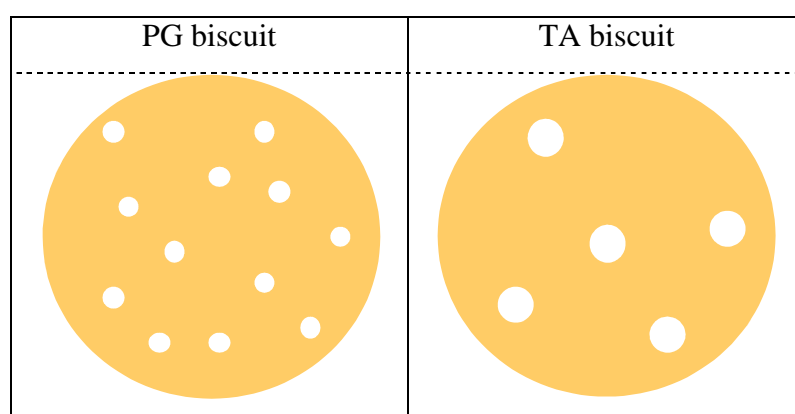
The cross sectional spatial porosity and pore size was compared to the absolute vanillin level distribution; the middle layers of biscuits had significantly more vanillin ( $p < 0.05$ ) with significantly more large pores ( $p < 0.001$ ). However, Pearson's correlations with two-tailed test indicated that there was no significant correlation between vanillin concentration with average porosity ( $p > 0.05$ ) or with average pore size ( $p > 0.05$ ), in both PG and TA biscuits.

Image analysis was applied for the two biscuit cutting approaches and compared with the results of vanillin distribution. A significant positive correlation was

found between vanillin concentration and the average porosity of seven cross sectional slices of the PG biscuits. When looking at all the data together there was no significant correlation between vanillin concentration and average pore size or porosity. However, image analysis provided a valuable tool to visualise the internal biscuit structure and may explain the effect of flavour solvent observed in previous storage studies.

#### 7.4.4 Impact of Solvent (Biscuit Structure Analysis)

The microstructure of food products has a large impact on the distribution of physical, mechanical and sensorial properties distribution (Baltsavias et al., 1999; Pages et al., 2007). The key finding from biscuit structure analysis was that PG biscuits had smaller pores and higher porosity than TA biscuits, which is schematically shown in Figure 7.27. The differences on biscuit structure between PG and TA biscuits are magnified, to help explain the texture and aroma differences found in biscuits with these two solvents.



*Figure 7.27 Schematic diagram illustrated PG biscuit structure with smaller pores higher porosity and TA biscuit structure with larger pores lower porosity*

From texture measurement results, TA biscuits were significantly more brittle, which might be due to larger pores joining through fracture lines (Figure 7.27) resulting in a shorter distance to product fracture and a smaller fracturability (mm)

being measured. Aroma compounds might be either adsorbed at fat phase or bound with the starch phase (Chevallier et al., 2000a). With a greater number of pores distributed in the PG biscuits, aroma compounds might be more dispersed throughout the biscuit matrix around these pores, due to the increased surface areas they would more easily transfer from matrix into the pore gas space and therefore be lost through internal channels between pores. In addition, the transport of vanillin through the hydrophobic matrix of TA biscuits would be more restricted if the aroma compounds are more tightly bound to the matrix due to elevated matrix hydrophobicity resulting in a greater retention of aroma in TA biscuits. This might explain the higher vanillin and HMF concentration in TA biscuits when only physical stability is considered.

The difference observed between PG and TA biscuits might be caused at the flavour mixing stage. The dough was prepared by blending fat base, flour and water base together (see page 125), and then separated into half with PG and TA flavourings added into the respective dough by 2 min blending. Since mixing of flavourings was at the late stage of dough preparation, it is possible that the insufficient flavouring incorporation causes inhomogeneous distribution within the dough. Therefore, it will be useful in future to investigate the impact of flavour solvent with different flavour incorporation methods, i.e., i) mixing these flavourings into the fat phase first then adding flour and water base; ii) mixing these flavourings into water phase first then flour and fat base. This might help understand whether different mixing methods would have an impact on flavour incorporation and thus cause different flavour solvent effects.



## **Part IV General Discussion and Conclusion**

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### **8 GENERAL DISCUSSION & CONCLUSION**

This thesis discussed two areas of study in order to understand two major issues faced by the flavour industry: i) flavour reformulation for products with different fat levels and ii) flavour stability within a product during storage.

#### **8.1 Flavour Reformulation Study**

Both instrumental and modelling approaches were successfully applied for flavour reformulation.

##### **8.1.1 Instrumental reformulation approach**

Regarding the objectives for this study (Section 2.2.1), the level of variation related to the instrumental reformulation approach for confectionery products was defined. Instrumental variation of 3% indicated the reproducibility of the APCI-MS measurement. Production variation of 21% (chewy candy) and 51% (pectin jelly) was due to the nature of confectionery manufacturing. Panellist variation during consumption of chewy candy and pectin jelly was 20% and 31%, which is inevitable due to individual physiological differences. These defined levels of variation were helpful to understand the constraints of the whole instrumental reformulation approach and to ensure that quality data can be produced in the following experiment.

Product variation was difficult to reduce due to the natural flavour mixing process with candy mass, but several batches of samples were combined to minimise the inter-batch variations. The major challenge to reformulate flavouring through in-vivo analysis for volatiles release in mouth during mastication was the variation

between panellists during eating of products. To minimise this in-vivo variation, more panellists with fewer replicate samples was compared to fewer panellists with more replicates. Five panellists performing three replicates were found to be the minimum requirement based on the approach of cumulative mean analysis. This protocol was then applied to reformulate flavourings through instrumental approaches. The cumulative mean, as a statistical analysis tool is normally applied in financial systems to smooth price data to identify underlying trends (Hogg et al., 2012). It was used in our study to identify the point when additional data had no additional statistical benefit to the trend line. This was a novel approach to solving flavour release variation issues during food oral processing. For example, when compared with previous reported work by Shojaei et al. (2006a) using 90 people with quadruplicate samples, the practicability results of five panellists consuming three replicates is much simpler and easier to apply in day-to-day commercial practice (Yang et al., 2011).

The feasibility of reformulating a real commercial flavouring with a real food product by instrumental reformulation was evaluated. A strawberry flavouring consisting of nine aroma compounds was applied into a 8% fat chewy candy (C) and a fat-free pectin jelly (P) with the aim to have similar aroma release profile. APCI-MS in-nose analysis was used to measure the flavour release differences during both samples, the release ratio (P/C) was then generated and used to reformulate the flavouring to minimise the differences in release between the two products.

Sensory tests showed that the reformulation was successful and that a significant improvement in overall strawberry flavour ( $p < 0.001$ ) was perceived by panellists.

Additionally, descriptive analysis showed that the strawberry flavour profile of reformulated flavour in chewy candies was much closer to the pectin profile in terms of sourness, fruitiness and juiciness. A similar instrumental reformulation approach was demonstrated previously on a single aroma compound in a simple milk system (Shojaei et al., 2006a). This study illustrated how this technique can be applied into real commercial flavourings with complex food products, for the first time.

#### 8.1.2 Modelling reformulation approach

A mathematical modelling approach was also taken (Section 2.2.2), the approach was carried out in a yoghurt base with different fat contents using an experimental flavouring - KTP4 which contained six aroma compounds with a range of Log P values. The model equation developed by Linforth et al. (2010), was used to predict release ratios between any two products with known fat contents. The calculated results proved that higher Log P compounds (more hydrophobic) were affected to a larger extent by the fat in the product than the hydrophilic compounds ( $\text{Log P} < 2.0$ ). This is the first study that demonstrated the relationship between compounds with different Log P values and the predicted lipid effects: the higher the compound Log P value, the larger the lipid effect.

The predicted release differences between 0.1% fat and 4.2% fat yoghurt were used to reformulate the KTP4 flavouring, which was then added into 0.1% fat yoghurt (LF) to target the aroma profile of 4.2% fat yoghurt (HF) with the original KTP4 flavouring. The in-vivo release of the KTP4 flavouring from LF/HF yoghurt was determined to show the effectiveness of the reformulation. A good correlation was found ( $r = 0.95$ ) between the measured ratios and the model's

predicted values, so the model was validated to give good predictions for what happened during in-vivo analysis. The second experiment measured the release difference between reformulated LF yoghurt and original HF fat yoghurt, and no significant difference ( $p > 0.05$ ) was observed, so the reformulated low fat yoghurt had a similar release profile as its high fat version. This demonstrates the accuracy of the model's prediction and its applicability to reformulate flavourings for complex food systems.

The model's reformulation was validated by sensory testing. Four commercial flavourings were reformulated for the low fat yoghurt, and the sensory results showed that only the reformulated banana flavouring was perceived to be significantly different ( $p < 0.05$ ) from its original flavouring in low fat yoghurt. The modelling reformulation might not be valid for strawberry, blackcurrant and coffee flavouring. This might be due to the hydrophobicity of these flavourings were less hydrophobic as the banana flavouring, and the lipid effect might not be perceivable between the original and reformulated flavourings. Although there was a limitation of the model's application, this is the first time an attempt has been made to apply this model reformulation approaches to real commercial flavourings in real food products.

### 8.1.3 Application of two reformulation approaches

To sum up, both instrumental and modelling approaches were proved to be valid approaches to reformulate real flavourings in complex food matrices. Compared with traditional reformulation by trial and error, either instrumental or modelling reformulation offers a more efficient and more accurate approach that can be applied into a wide range of products. For example, reformulating through trial

and error might take several weeks for the flavourists and food technologists to work together until the required flavour is obtained in the final food product, but the instrumental approach can get accurate ratios to reformulate within a couple of days, and the modelling approach would take just a minute to calculate the reformulation factors from the equation, which can then be applied into that food product at the designated fat level.

The cost of raw materials can be reduced, not only through fewer trials applied during reformulation, but also through cost in-use. The reformulated flavourings at a higher quality developed by either approaches could be saved up to 20-30% of the flavourings in-use.

The results obtained from reformulation studies have been presented to the major customers of Aromco and some potential clients worldwide. A range of commercial flavourings up to 40 types were selected with a reformulated version for chewy candy based on the model's prediction, and five of them have already been successfully delivered to Aromco's customers. As a result, the sales and reputation for Aromco's innovation and technology advance was found to be increased considerably at the end of this project.

With the understanding of the advantages and limitations for both approaches (Section 4.3.2.), they are presented as tools to help flavourists formulate flavours for different food matrices. Further improvement in flavour quality can be gained using the skills and experience of flavourists to "round out" the reformulated flavourings (Yang et al., 2011).

## 8.2 Flavour Stability Study

Although flavourings, as it has been shown in Section 8.1, can be effectively reformulated for food products with different fat contents, their desirable flavour profile might change significantly during storage. The aim of the later phase of the study was to increase our understanding to help maintain flavour in food products. The investigation was focused on vanillin stability within stored biscuits, whilst also comparing two flavour solvents (PG and TA).

### 8.2.1 Instrumental analysis

Prior to this study, the preliminary test was carried out to select biscuits with minimum variation after baking. The results of colour and texture measurement indicated larger variations at the edge of the baking tray, so these biscuits were excluded in later experiments to minimise the overall product variation. Without the additional vanillin, TA biscuits were significantly less yellow, more brittle, and had higher HMF concentration than PG biscuits and biscuits with no flavour solvent ( $p < 0.05$ ). This preliminary finding highlighted the possible impact of solvent choice on biscuit colour, texture and aroma, and identified baking variation at the edge of the tray, which was avoided to ensure quality data can be produced in the later studies.

During eight weeks ASL test, the changes of vanillin in the stored biscuits were investigated. The instrumental results showed up to 20% vanillin reduction during the eight weeks of the ASL test at three storage temperatures (20, 32.5 and 45 °C). The vanillin concentration in all the biscuit samples was reduced with time ( $p < 0.001$ ) and with elevated temperatures ( $p < 0.05$ ) significantly. Previously, Anuradha et al. (2010) found up to 16.6% vanillin lose in biscuits after 60 days

storage at 38-40°C, which is consistent with our findings and therefore validates our ASL approach.

Besides vanillin, the changes other aroma compounds was measured during the ASL test. The HMF concentration representing the biscuit aroma was found at significantly lower levels with the increased storage time ( $p < 0.05$ ). The loss of HMF is postulated to be due to its volatility, but the loss between the three storage temperatures had no significant difference ( $p > 0.05$ ). This might be because HMF was formed at a much higher temperature during the Maillard reaction or Caramelisation reaction (Kroh, 1994). In addition, the oily off-note generated from lipid oxidation in the stored biscuits were represented by 2,4-decadienal and 2,4-heptadienal. The analysed results indicated that their concentrations increased significantly with storage time ( $p < 0.001$ ), and a significantly higher amount was formed at 45 °C than at 32.5 °C and 20 °C ( $p < 0.001$ ), which highlighted the need to control lipid oxidation during the stored biscuits.

Due to a dramatic increase in price for most raw materials in the flavour and food industry, an understanding of the flavour stability (particularly vanillin) within stored products is especially beneficial for flavour companies to ensure their high quality materials are not only delivered to their clients but are also maintained in the food products after it has been in storage for the end users.

In addition to aroma analysis, the changes of biscuit texture during the ASL test were also evaluated. The results demonstrated no significant change for either hardness or fracturability with storage time ( $p > 0.05$ ). However, temperature did have a significant impact ( $p < 0.001$ ), biscuits stored at lower temperature (20 °C)

were significantly less hard and less fracturable than those stored at higher temperatures (32.5 °C and 45 °C).

The aroma and texture differences between TA and PG biscuits were compared. PG biscuits exhibited greater losses in vanillin generally and the HMF concentration was significantly higher in TA biscuits ( $p < 0.001$ ). The solvent effect was also significant for the two oily aroma compounds (2,4-decadienal and 2,4-heptadienal), i.e., a significantly lower level for both compounds was observed in TA biscuits ( $p < 0.001$ ). In terms of texture, the hardness between the two types of biscuits were not significantly different ( $p > 0.05$ ), but TA biscuits were significantly more prone to fracture than PG biscuits ( $p < 0.05$ ), which was in agreement with previous findings in the preliminary studies. The impact of solvent on biscuit aroma and texture might be due to their different physicochemical properties like boiling point, volatility and hydrophobicity. This is the first study to compare the use of two different flavour solvent on the effect of biscuit texture and aroma and demonstrates that choice of flavour solvent can have a impact both the final product and shelf-life stability, which of significance widely across the flavour industry.

Finally, the objective was achieved as the results showed that biscuits at normal storage (20 °C, 24 week) had similar vanillin reduction level (14%) compared to the 13% reduction in the ASL stored biscuits (45 °C, 8 week). However, the normal stored biscuits had significantly lower levels of 2,4-decadienal generated than the ASL stored biscuits ( $p < 0.05$ ). This study showed that ASL test might not fully predict the changes in normal conditions, but it can be used as a quick



tool to instrumentally identify some of the major storage changes that may occur in the final products.

### 8.2.2 Sensory analysis

Sensory analysis was compared with the instrumental analysis for the ASL stored biscuits. The results from the paired comparison tests illustrated: i) no significant difference in terms of perceived brittleness (fracturability) ( $p > 0.05$ ), which correlated with instrumental results; ii) no significant perceivable vanilla flavour difference for all stored biscuits ( $p > 0.05$ ), although instrumental analysis showed up to a 20% vanillin reduction; iii) and that the oily off-note was not significantly perceived for stored PG biscuits, but it appeared to be perceivable in TA biscuits stored after 6 weeks ( $p < 0.05$ ), a difference which was not apparent after 8 weeks storage. As a result, the changes observed by instrumental analysis might be either at sub-threshold level (i.e., below the just noticeable difference) or undetectable by the sensory approach taken in this study.

The sensory changes for biscuits at a normal storage test (24 weeks, at 20 °C) were compared with these in the ASL conditions. The results of the normal stored biscuits indicated that i) no significant difference was perceived on biscuit brittleness ( $p > 0.05$ ); ii) vanilla flavour was perceived as significantly weaker for normal stored PG biscuit ( $p < 0.05$ ); iii) no significant oily off-note was perceived at this normal condition ( $p > 0.05$ ). The results of brittleness and oily off-note perception were similar to the ASL sensory findings, but vanillin flavour perception differed between normal stored biscuits and the ASL stored biscuits. Previous instrumental analysis suggested the similar level of vanillin reduction

between the ASL and normal storage conditions, so further sensory study was applied in the following.

Further sensory tests were carried out to determine the detectable difference level of vanillin in fresh biscuits. Biscuits with the original vanillin level were compared to those with reduced vanillin level from 10%, 20% to 30%, and the triangle test results indicated that there was no significant sensorial difference at all reduction levels ( $p > 0.05$ ). These findings confirmed the sensory results for the ASL stored biscuits, but it was difficult to explain the significant difference perceived in normal stored biscuits. In future studies, it is necessary to either repeat the experiments with a larger number of panellists or use alternative method in order to confirm the level of detectable difference for vanillin in biscuits.

The above results indicated that small changes in vanillin concentration (20%) were undetectable, and therefore it is important to validate instrumental findings with sensory evaluations.

### 8.2.3 Structure analysis

The micro-structure of PG and TA biscuits was compared by X-ray analysis. The results illustrated that TA biscuits had significantly larger pores ( $p < 0.001$ ) and a smaller number of pores ( $p < 0.001$ ) than PG biscuits. The novel approach to study biscuits using X-ray analysis was previously reported by Frisullo et al. (2010), but the impact of solvent on biscuit micro-structures as measured by X-ray analysis has never been reported in the literature and has potential to be applied to many food systems.

In addition, aroma distribution inside biscuits at different sections was evaluated. The analysis results indicated higher vanillin concentration in the centre of biscuits than the edges ( $p < 0.05$ ), and HMF concentration was higher at the surface of the biscuits ( $p < 0.05$ ). A significant impact of flavour solvent ( $p < 0.05$ ) was confirmed: TA biscuits had significantly higher vanillin and HMF concentrations than PG biscuits.

Finally, the differences in the biscuit structure helped to explain the texture and aroma difference between PG and TA biscuit in the following ways. TA biscuits were more fracturable because of some larger pores present at the breaking point causing a relatively shorter distance to break. PG biscuits had a larger number of smaller pores distributed within the matrix, causing aroma compounds to be more dispersed around these pores and more easily transferred into the air. Consequently aroma compounds might be less stable in PG biscuits. These pores might even make the lipid more physically available to oxidation, explaining why more lipid off-notes were generated in PG biscuits.

The result of TA flavouring offering better flavour stability than PG flavouring in biscuits can be beneficial for flavour and bakery companies, especially those who sell to consumers in hot countries like Africa and the Middle East. The cost of these two solvents has been comparable since 2011 (Brown, 2011), so using TA instead of PG for flavourings in bakery products could offer better stability at the same cost.

In conclusion, the main novelty in this work lies in combined use of structural analysis, chemical profiling and sensory testing to elucidate new approaches (variability) and new tools (flavour solvent) to enhance methodological

reformulation approaches and flavour stability in real foods. Both flavour reformulation study and flavour stability study proved that it is feasible to apply laboratory-derived knowledge and adapt scientific techniques in a commercial context. It has also demonstrated how the research findings can be transferred into the commercial field.

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